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## Detailed follow-up of hepatitis B surface antigen positive blood donors.

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The Open University.

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Faculty of Biology.

Dissertation for the Degree of Bachelor of Philosophy.

Susan Brown

North London Blood Transfusion Centre

Colindale.

Detailed follow-up of hepatitis B surface antigen

positive blood donors.

May 1992.

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## Glossary of abbreviations.

AFP	alpha-foetoprotein
ALT	alanine aminotransferase (see also GPT).
Anti-HBc	antibody to hepatitis B core antigen.
Anti-HBs	antibody to hepatitis B surface antigen.
Anti-HBe	antibody to hepatitis B e antigen.
AST	aspartate aminotransferase (see also GOT).
az.	sodium azide.
BPL	Blood Products Laboratory.
BSA	bovine serum albumin.
°C	degrees centigrade.
CMV	cytomegalovirus.
cpm	counts per minute.
DHBV	Pekin duck hepatitis B virus
DNA	deoxyribonucleic acid.
EIA	enzyme immunoassay.
ELISA	enzyme linked immunosorbent assay.
EBV	Epstein-Barr virus.
GOT	glutamic oxaloacetic transaminase (see also AST)
GPT	glutamic pyruvic transaminase (see also ALT).
GHSV	ground squirrel hepatitis virus
HA	haemagglutination.
HAV	hepatitis A virus.
HBV	hepatitis B virus.
HBcAg	hepatitis B core antigen.
HBeAg	hepatitis B e antigen.
HBsAg	hepatitis B surface antigen.

HSV	herpes simplex virus.
IgG	immunoglobulin G.
IgM	immunoglobulin M.
ID	immunodiffusion
LFT	liver function test.
IRMA	immunoradiometric assay (see SPRIA)
min	minute.
ml	millilitre.
ul	microlitre.
NLBTC	North London Blood Transfusion Centre.
NHS	normal human serum.
ng	nanogram.
nCi	nanocurie.
%	percent.
PCR	polymerase chain reaction.
PHC	primary hepatocellular carcinoma.
PTH	post transfusion hepatitis.
RIA	radio-immunoassay.
RNA	ribonucleic acid.
RPHA	reverse passive haemagglutination.
sal.	saline.
SPRIA	solid phase radio-immunoassay (commonly used also for IRMA but technically refers to the competitive assay).
THBV	tree squirrel hepatitis B virus
Tris.	tris-hydroxymethylaminomethane.
WHO	World Health Organisation.
WHV	Woodchuck hepatitis virus.

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### Abstract.

Although the North West Thames Region collects over 200,000 blood donations each year, less than four cases of post-transfusion hepatitis B are reported to us annually (Barbara and Briggs 1981) presumably reflecting the exclusion of approximately 40 donors per annum found to be Hepatitis B surface antigen (HBsAg) positive. This study is the first step in the analysis of some of the unique data accumulated on these HBsAg-positive donations over an eighteen year period.

Routine HBsAg screening of all blood donations became mandatory in the U.K. by 1971. Since then, we at the North London Blood Transfusion Centre (NLBTC) have monitored as many HBsAg-positive donors as possible to obtain epidemiological data on HBsAg carriage and the prevalence of other hepatitis B virus (HBV) markers, and to examine ways of predicting the course of HBV infection, so that we can advise carriers concerning their level of infectivity and management of their infection in everyday life.

The first section examines the role of HBV in the context of blood transfusion. The serological markers of HBV infection are described in detail, including viral subtypes and a description of mutant and defective strains. The HBV carrier state is then briefly described.

The second section describes current microbiological practices in blood transfusion including the present trend towards laboratory automation.

The third section details the serological assays available for the laboratory detection of HBV infection and an experimental evaluation defines the confidence we can place upon assay results.

Section four is a preliminary analysis of some of the epidemiological data accumulated at the NLBTC over 18 years. 286 HBsAg carriers have been followed up; with particular attention to their HBe antigen and antibody status as the markers most relevant to defining infectivity levels. Of the 35 carriers who were HBeAg-positive at the time of detection, 8 seroconverted from HBe antigen to anti-HBe during the follow-up period. HBeAg positivity was shown to be associated with raised liver function test (LFT) values. Those who seroconverted from HBe antigen to anti-HBe eventually developed normal LFT levels. My preliminary observations on the duration of HBsAg positivity and also on temporal changes in HBsAg titre will form the basis of further work. Although complete in itself, this study also serves as a pilot for a more comprehensive analysis of the wealth of material still to be examined.

## Introduction.

I have been employed as a clinical scientist at the North London Blood Transfusion Centre since 1979. During that time most of my work has been concerned with the screening of blood donations for hepatitis B surface antigen and other serological markers of infection with the hepatitis B virus. I have also been responsible for keeping a complete record of all hepatitis B surface antigen-positive donations made at the centre, both at initial recognition and later as a result of the examination of confirmatory and follow-up samples, often over a long period of time. The archive material accumulated during the course of my work is probably one of the most comprehensive collections available worldwide on hepatitis B detection in donated blood. This thesis is concerned with the analysis of some of the data, together with a description of experimental work directed at defining the precision and improving the reliability of the testing procedures.

In section 1, I introduce the hepatitis B virus in the context of blood transfusion. The section starts with a description of the virus and the disease to which it gives rise in man: this is followed by a description of the relationship of the hepatitis B virus to other hepatitis viruses of man and of certain animals. I then describe all the relevant serological markers of infection with the hepatitis B virus and show how the virus exhibits different subtypes which are useful epidemiologically in following

the spread of infection. The existence of mutants of the virus and their probable natural selection to evade the immune response after vaccination are discussed. Finally I examine the intriguing phenomenon of the existence of a carrier state for the virus.

Section 2 contains an outline of the practice of transfusion microbiology with particular reference to the North London Blood Transfusion Centre where this study was carried out. Current techniques are described in some detail but these are likely to be improved and replaced in future years, therefore I also include a section on the role of automation in transfusion microbiology as this is one likely direction of future development.

Section 3 describes the assays available for the detection and quantitation of the important serological markers of HBV infection, and includes an evaluation of the main tests used to amass the data employed in this study. A separate sub-section summarises the results of a comparative study of some of the methods currently available for HBsAg screening. Full protocols for the assays used are given as appendices.

Section 4 consists of a preliminary analysis of the wealth of epidemiological data accumulated at NLBTC over the last 18 or more years. I attempt to analyse trends in the donor population and pay particular attention to the significance to the donor of his infectivity status and also to the likely future course of his disease. Other workers have

attempted to answer these or similar questions but nowhere has there been such an extensive study in terms of the very large numbers of donor samples and length of follow-up available at NLBTC and the conclusions reached as a result of my work add significantly to an understanding of the importance to blood transfusion science of the hepatitis B virus.

This study is complete in itself but much material remains to be examined. The work now completed will therefore serve as a pilot for an even more comprehensive analysis at a future date.



**SECTION 1 : HEPATITIS AND HEPATITIS VIRUSES.**

### 1:1 Viral Hepatitis.

Viral hepatitis is a systemic disease caused by one of several viral agents showing a predilection for the liver.

The clinical picture of hepatitis infection may vary in its presentation from inapparent or subclinical infection, slight malaise, mild gastrointestinal symptoms and the anicteric (not exhibiting jaundice) form of the disease, through the acute icteric (with jaundice) illness, severe prolonged jaundice and chronic liver disease, to acute fulminant hepatitis. (See below).

The acute anicteric form of the disease is characterised by malaise, anorexia, nausea and various gastrointestinal symptoms. Fever may sometimes occur. Clinically, the liver is enlarged and tender, bile is present in the urine and biochemical tests show elevation of serum transaminases and a transient rise in conjugated bilirubin. The mild nature of the clinical manifestations does not preclude progression of the hepatic lesions to chronic damage of the liver.

In the case of acute icteric hepatitis, the duration of symptoms in the pre-icteric phase of the illness varies from 1-14 days, but may be longer. Marked anorexia and nausea are particularly prominent. Headache, generalised myalgia, arthralgia, skin rashes and fever may be present

in a number of cases. The predominant presenting symptoms are gastrointestinal and toxic in nature. The pre-icteric stage progresses to the icteric phase with the appearance of dark urine, clay-coloured stools and jaundice. With the appearance of jaundice there is usually a rapid improvement in symptoms. The jaundice usually deepens during the first few days and persists for one or two weeks. The faeces then darken again and the jaundice gradually diminishes.

Recurrence of symptoms and relapses with jaundice may occur and sequelae such as subacute hepatitis, chronic hepatitis and post-hepatic cirrhosis may follow.

In 1946 Lucke and Mallory introduced the term "fulminant hepatitis" to describe a rare form of viral hepatitis which was described in the past as "acute yellow atrophy of the liver". This form of the disease has a very high mortality rate and death often occurs within ten days of onset as a result of massive necrosis of the liver. Progressive deep jaundice and widespread haemorrhages are a constant feature. Stupor, hepatic coma and death usually follow.

During the past two decades, there has been vast progress in our understanding of the disease and its causative agents. At least four types of viral hepatitis are recognised today. These are hepatitis A, hepatitis B, hepatitis C, delta hepatitis and non-A, non-B (NANB) hepatitis. In addition, sporadic hepatitis may be caused by other viruses, for example, yellow fever virus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), rubella virus and the enteroviruses.

A variety of agents have been implicated in the transmission of viral hepatitis, the most common being the hepatitis A virus (HAV), which causes short incubation hepatitis, and the hepatitis B virus (HBV), which is associated with long incubation or "serum" hepatitis. The empirical separation of acute viral hepatitis into two forms (short and long incubation) was the result of epidemiological data amassed by various workers 1944-1951 (see A.J.Zuckerman 1972 ) and led to the discovery of the "Australia antigen", now recognised as hepatitis B surface antigen, in the 1960s by Baruch Blumberg. The actual hepatitis B infective virus particle was described by Dane et al. in 1970.

In the mid-1970s improved ability to diagnose hepatitis A and hepatitis B led to the recognition of the existence of yet another type of viral hepatitis, this was designated non-A, non-B hepatitis. For a long time, the causative agent(s) of both blood-borne and water-borne hepatitis NANB were unknown, but recently, in 1989, the Chiron Corporation of California USA, announced the discovery and cloning of the agent implicated in the transmission of the major blood-borne variety of the disease now known as the hepatitis C virus (HCV). A viable laboratory screening test has been developed and it is now possible to provide blood screened for all known agents of viral hepatitis including HCV.

One of the most severe forms of human viral hepatitis (especially when associated with chronic hepatitis B) is Delta hepatitis, which is one of the more recently recognised forms of the disease. The Delta agent (HDV) is a defective virus which requires the presence of the hepatitis B virus to provide its coat antigen. HDV is a cytopathic virus giving rise to clinical and often severe hepatitis.

In blood transfusion, the most reliable method for preventing HBV transmission is the screening of blood donors for the presence in their serum of the major coat-protein of the virus, the hepatitis B surface antigen (HBsAg). This screening is now mandatory for all donor blood collected in the U.K. At the North London Blood Transfusion Centre, donors who are positive for HBsAg are never used for transfusion purposes but are followed up and monitored wherever possible to observe the course of their infection.

## 1:2 The Hepadna Viruses.

The hepadna viruses are hepatotropic viruses which include the human hepatitis virus (HBV), woodchuck hepatitis virus (WHV), duck hepatitis virus (DHBV), ground squirrel hepatitis virus (GSHV) and more recently, tree squirrel hepatitis virus (THBV). All these viruses cause persistent virus infection, but only HBV and WHV go on to cause chronic active hepatitis. Growing evidence suggests that localisation of the HBV antigens on the surface of infected hepatocytes represents a target for T-lymphocyte attack, causing lysis of autologous hepatocytes. The apparent failure to lyse infected hepatocytes, usually accompanied by normal liver morphology, is a characteristic attribute of the HBV carrier state and is also found in chronic carriers of woodchuck hepatitis virus. Chronic WHV infection of woodchuck appears to be associated with an active form of chronic liver disease and hepatocellular carcinoma (HCC). Thus the woodchuck has been an excellent animal model of human diseases such as acute and chronic viral hepatitis and HCC (Millman et al., 1984; Summers et al., 1980a).

The hepadna viruses contain small, circular DNA molecules that are partly single-stranded; a DNA polymerase in the virion can repair the DNA and make it double-stranded. In some cases the antigens of the non-human hepadna viruses within this group partially cross-react with those of HBV,

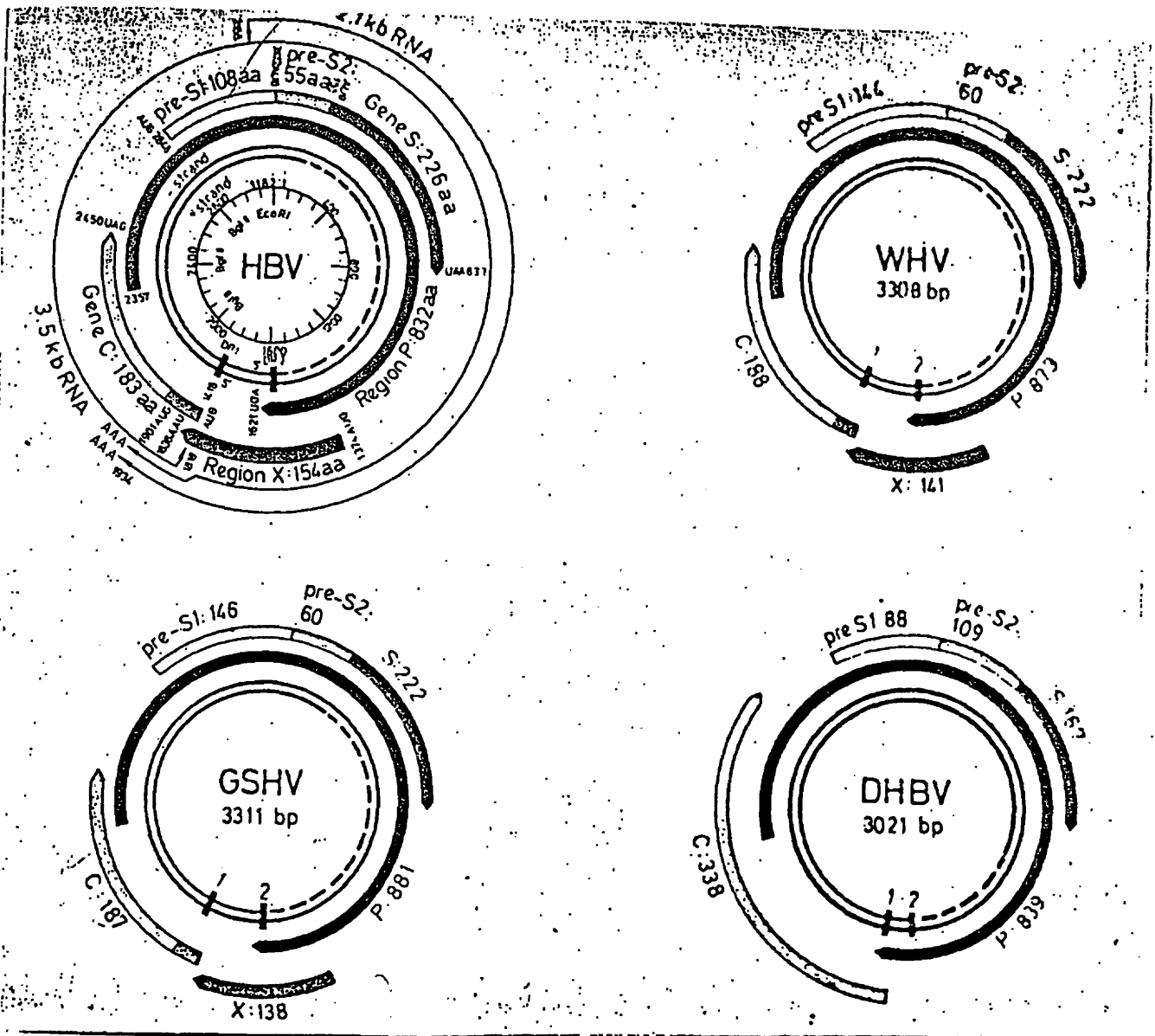
especially in the case of the surface and core antigens and respective antibody responses (Cote and Gerin, 1983; Gerin, 1979; Millman et al., 1982; Siddiqui et al., 1981) when detected in blood or liver tissue. Other viruses in this group are morphologically similar to HBV. (See figure 1 ). However it is to be noted that the Duck hepatitis virus does not possess the X-region in the genome.

Important biological features of the hepadna group of viruses include tropism for hepatocytes and very narrow host ranges. There is a strong association between long-standing hepadna virus infection and the development of primary hepatocellular carcinoma in both humans and in woodchucks, and viral DNA is found integrated in the cellular DNA of many tumours.

Superficially these viruses appear to share some features with RNA tumour viruses or retroviruses. Among the common features are similarities in genome structure, (see figure 1) although the nucleic acids in their virions are different, i.e. RNA in retroviruses and DNA in hepadna viruses, hepadna viral DNA appears to replicate through an RNA intermediate using a reverse transcriptase analagous to retrovirus replication. Viruses of both groups cause persistent infection and viral antigen can be carried in the blood for many years. Another similarity between them is that viruses of both groups appear to integrate readily in cellular DNA . Two well-studied PHC cell lines (PLC/PRF/5 and Hep 3B) producing HBsAg have been shown to have integrated HBV DNA sequences, integration of HBV DNA

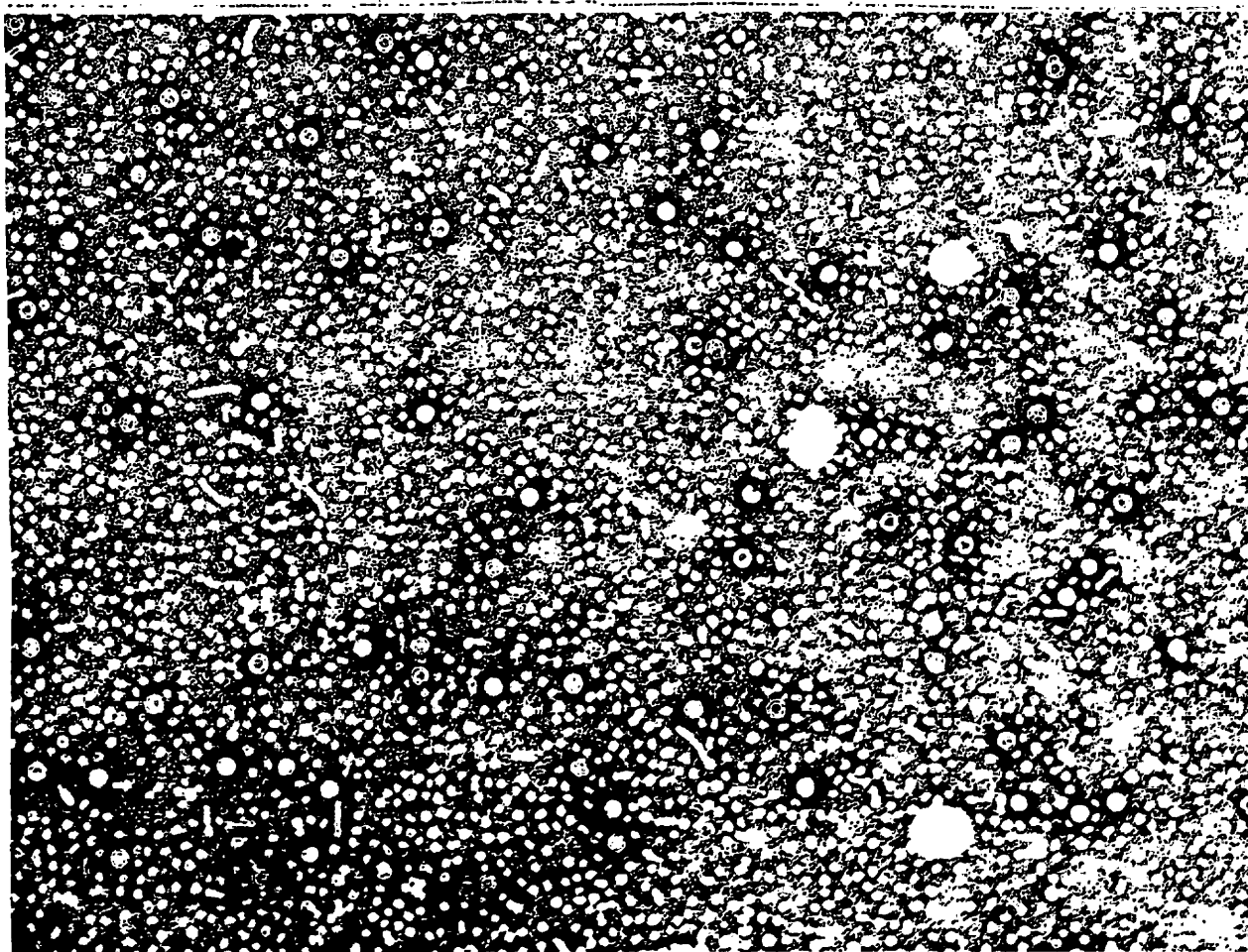
when it does occur, is early in infection (Brechot et al., 1981), but this integration has not yet been shown to be essential to the mechanism of cell transformation, as is the case in retroviruses. When exclusively integrated in the DNA of infected cells, both hepadna viruses and retroviruses may express only the gene for their envelope protein.





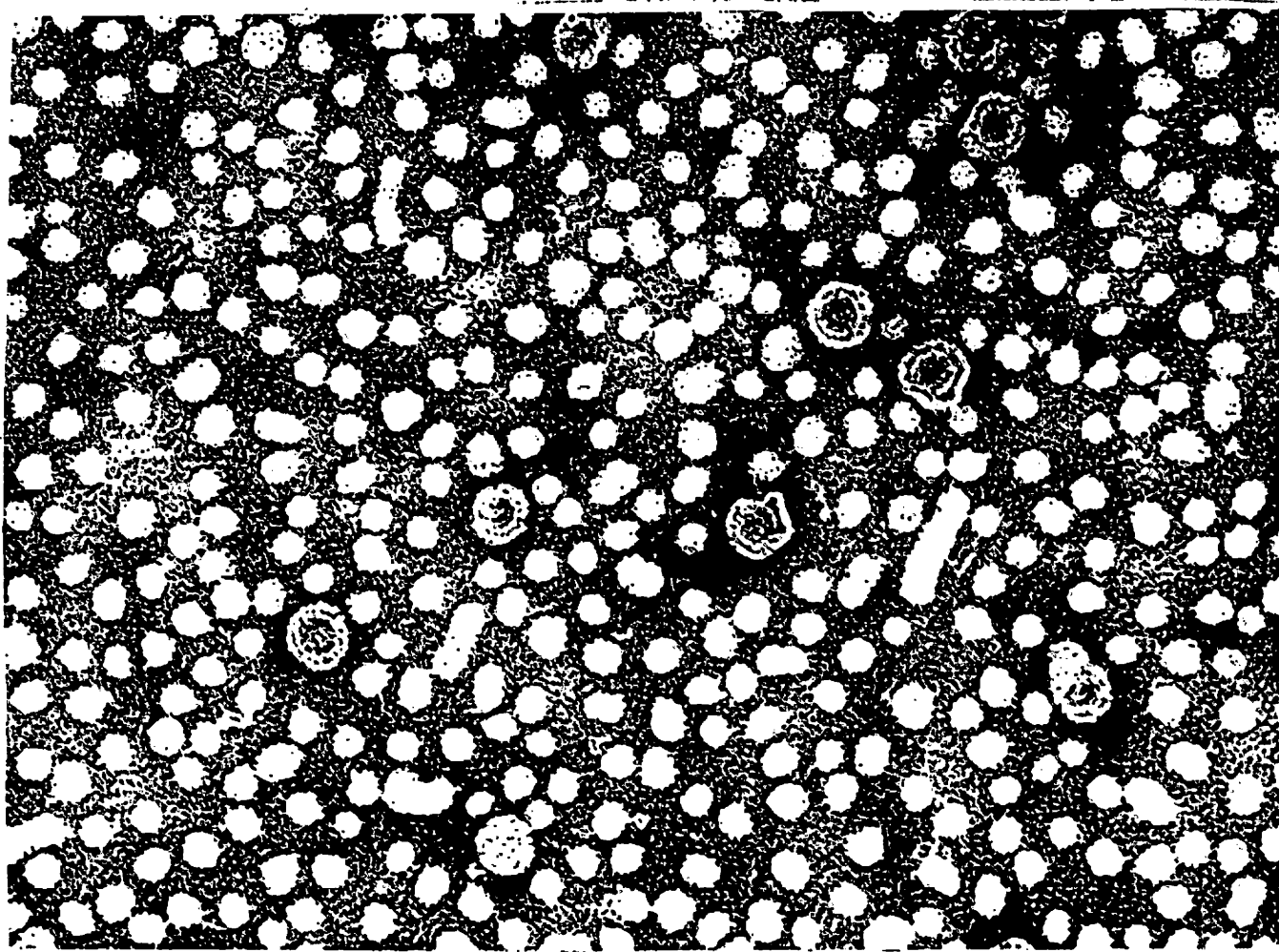
**Figure 1.**

Genomic organisation of the viruses in the hepadna virus group. HBV = hepatitis B virus, WHV = woodchuck hepatitis virus, GSHV = ground squirrel hepatitis virus, DHBV = Pekin duck hepatitis virus. C = core gene, S = surface gene P = polymerase gene, X region - function not known. Note the partially overlapping regions for different genes. This diagram shows the similarity of the genomic structure, which mirrors the morphology and also the pathology of the different agents in the different hosts. The shading relates to the different defined genes. ( see also figure 2. for genetic organisation of HBV.)



100nm

Plate 1. Electron Micrograph showing Hepatitis B Virus  
particles.  
(x 7000)



100nm

Plate 2. Electron Micrograph of HBV showing 42 nanometer  
particles (HBcAg) and 22 nanometer particles (HBsAg).  
(x 200,000)

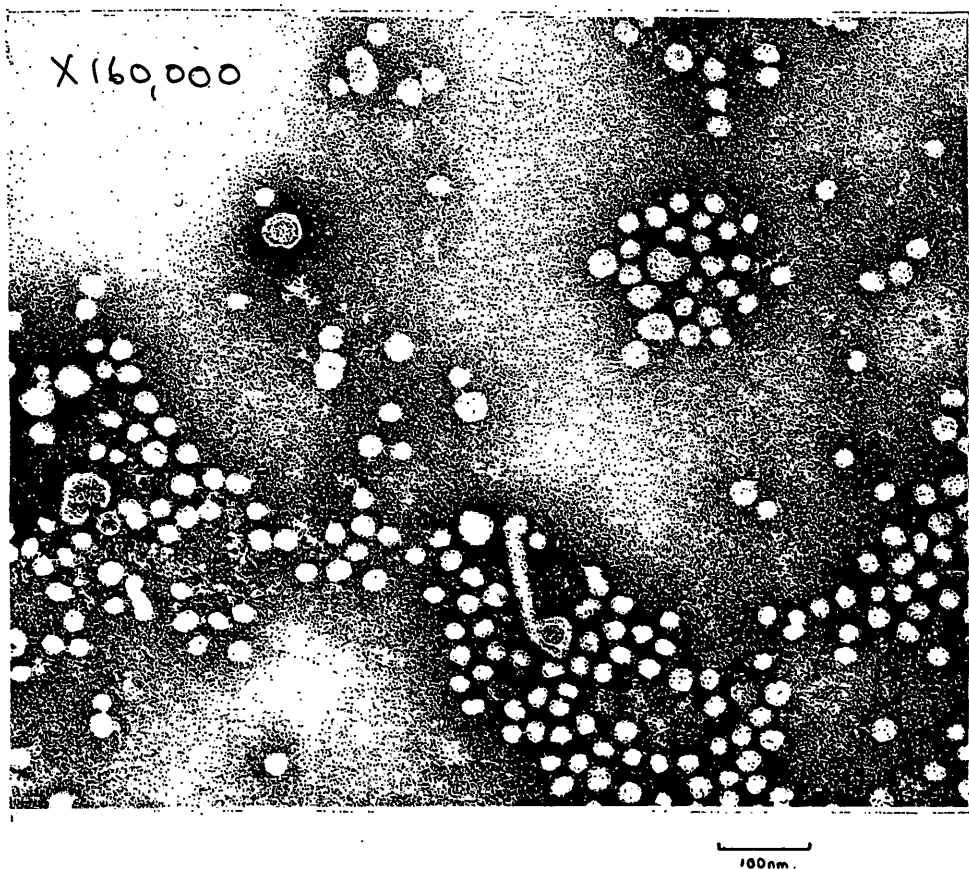


Plate 3. Electron Micrograph of hepatitis B Virus Particles

(x160,000).

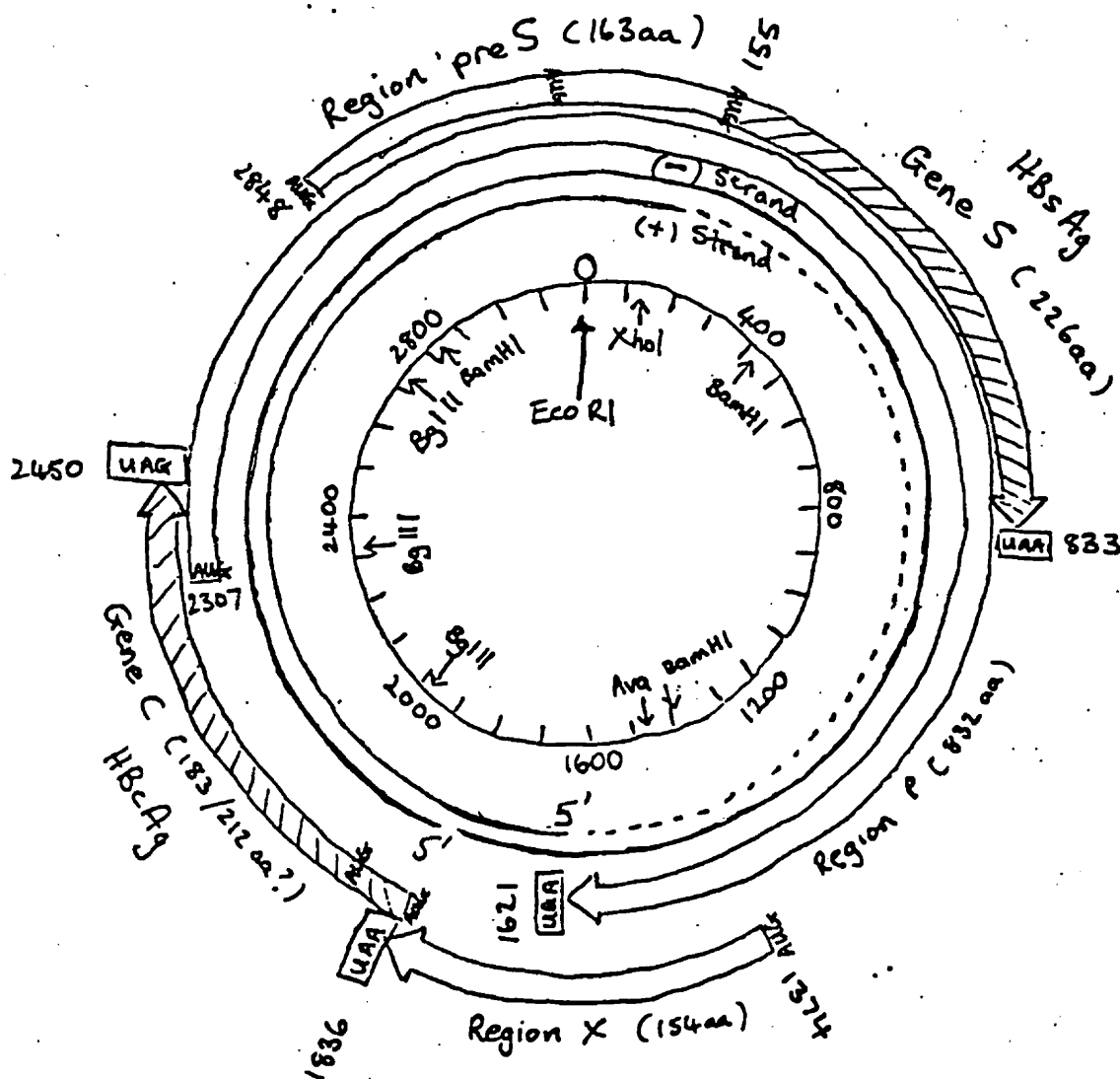
### 1:3 The Hepatitis B Virus.

The viral aetiology of hepatitis B was suggested in 1908 by McDonald but was established by transmission experiments on human volunteers during World War II. (Voegt 1942, Cameron 1943, MacCallum and Bradley, 1944). Evidence that a prolonged carrier state persisting for at least 5 years has been provided by Stokes et al. (1954), Neefe et al. (1954) and Murray et al. (1955a,b). Murray et al. (1954) recorded the case history of a donor in whose blood hepatitis B virus was found 135 days after he had donated blood which was subsequently incriminated as the cause of hepatitis in the recipient. The same donor later developed hepatitis himself. Viraemia was demonstrated once again, on this occasion 6 months later.

A marker for the presence of the causative agent was first discovered in 1964 when Blumberg was investigating genetically-determined serum protein polymorphism using the Ouchterlony technique of double diffusion in agar gel. He found that an antigen from the serum of an Australian Aborigine reacted with an antibody in the serum of a haemophilia patient who had received multiple transfusions. A year later, a technician in Blumberg's laboratory developed hepatitis and discovered the "Australia Antigen" in her own blood and subsequently developed viral hepatitis. This occurrence, and the same finding in a patient with Down's syndrome who developed hepatitis, provided information as to the cause of the disease.

Subsequent studies on patients with viral hepatitis, specifically hepatitis B, confirmed these findings, culminating in the clear linkage of HBsAg and hepatitis B infection proposed by Prince in 1968.

The actual virus was identified in 1970 by Dane et al., who described the morphological forms of the virus (see figures 3 and 4).



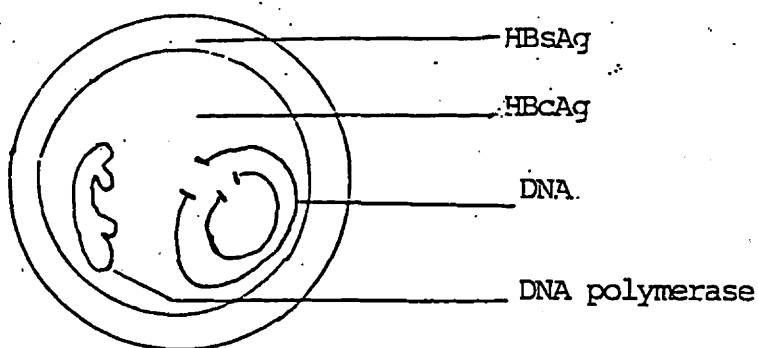
**Figure 2.**

Genetic organisation of HBV (c.f. figure 1).

The broad arrows surrounding the genome represent the four large open reading frames of the (-) strand transcript. The four potential coding regions are termed region S (gene S + region pre-S), gene C, region P and region X. The two regions corresponding to the defined genes S and C are represented by crosshatching.

The numbering of the nucleotides on the inner circle corresponds to the genome of the ayw<sub>3</sub> subtype.

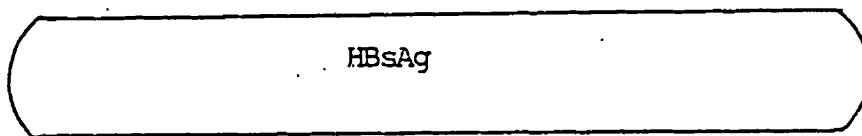
DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF THE HEPATITIS B VIRUS-ASSOCIATED PARTICLES.



"Dane" Particle - 42 nanometers.



Spherical Particle - 27 nanometers.



Filamentous Form - 120 x 22 nanometers.

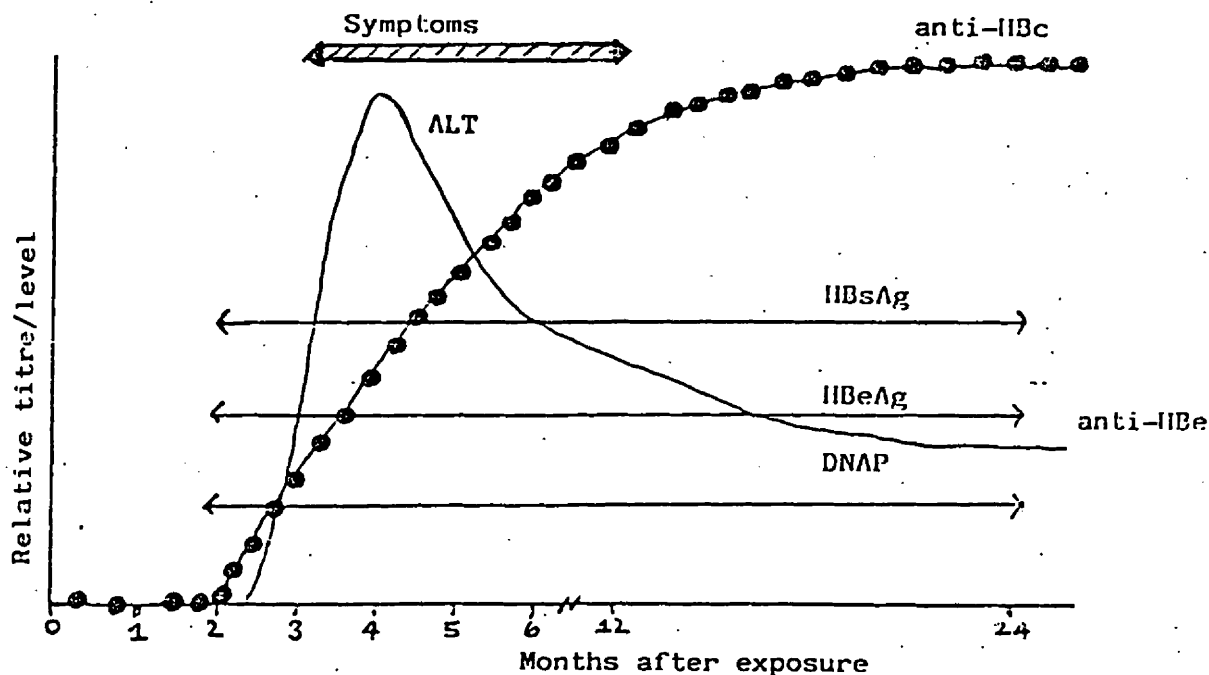
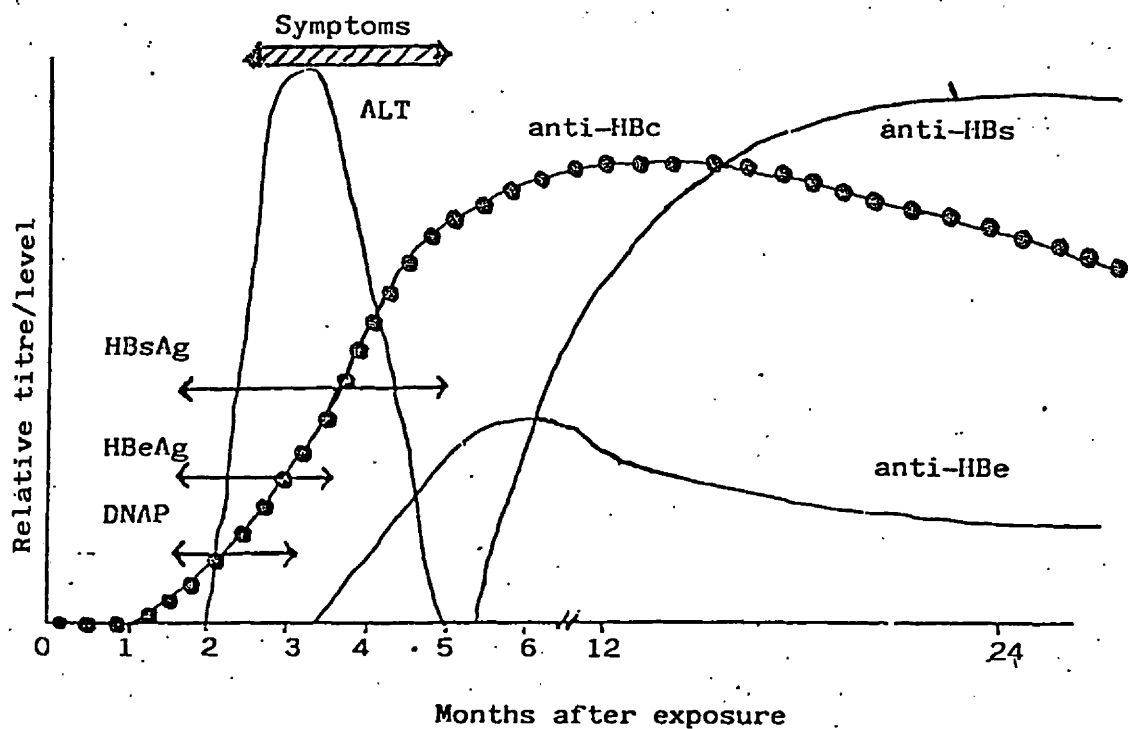
Figure 3.



Figure 4.

Summary table of HB virus particles.

	<u>Size</u>	<u>Name</u>	<u>Comment</u>
Large particles	42nm diameter	'Dane' particle	Virion containing double-stranded DNA
-----	-----	-----	-----
			Contains genome,
Nucleocapsid core	27nm diameter	core	always enclosed in Dane particle.
-----	-----	-----	-----
	18-22nm diameter	spherical	Excess surface Ag.
Small particles	diameter pleomorphic	'22nm particles'	No DNA present
-----	-----	-----	-----
Long particles	22nm diameter	tubular particles	?only present if Dane particles present.
-----	-----	-----	-----



**Figure 5.**

Diagrammatic representation of the serological responses in (above) acute hepatitis B infection and (below) in chronic hepatitis B infection. (From Gerety).

#### 1:4 Serological Markers of the Hepatitis B Virus.

HBV is an icosahedral particle 42 nm in diameter, enclosing a 27 nm electron-dense core (HBcAg) which contains circular DNA of low molecular weight.

Two distinct populations of HBcAg have been identified and separated by ultracentrifugation on the basis of their discrete densities. The heavier HBcAg particles (1.36 g/cm<sup>3</sup>) contain DNA and HBV-specific DNA polymerase, while the lighter particles are aggregates of surface antigen free of DNA.

Specific antibodies to HBcAg develop early during acute hepatitis B and persist for long periods in persons convalescent from hepatitis B. IgM anti-HBc appears early in acute hepatitis B, and IgG anti-HBc appears late in disease or during recovery and persists beyond convalescence.

One of the unique features of infection by hepadna viruses including HBV is the production of large quantities of excess viral coat protein referred to as hepatitis B surface antigen (HBsAg). This surface antigen is serologically identical to the Australian antigen. Surface antigen particles are 18-22 nm in diameter and are seen in both spherical and long tubular forms.

The presence of a DNA molecule in the Dane particle was first described by Robinson and colleagues (J.Virol 1974).

The structure of the HBV genome is characteristic and unusual. It is a small, circular, partly double-stranded DNA molecule with a single-stranded region of variable length. The long (-) strand is linear and of a fixed length of about 3,200 nucleotides. The short (+) strand is of variable length ranging from 50-75% of that of the long strand. The position of the 5' ends of the long and short strands and that of the 3' end of the long strand are fixed, while the position of the 3' end of the short strand is variable. The maintenance of the circular structure of the genome is assured by base pairing of the 5' ends of the two strands over a length of about 200 nucleotides. A DNA polymerase is associated with the capsid and a protein is covalently linked to the 5' end of the long strand. The activities of these two proteins seem to be related to viral replication. Like the other hepadna viruses, HBV is one of the smallest mammalian DNA viruses. By employing extensively overlapping regions the virus is able to encode much more protein than its genome length would otherwise allow.

Gel electrophoresis of HBsAg reveals two major proteins with molecular weights of 25,000 and 28,000 and at least four other proteins with molecular weights greater than 25,000. All of these proteins appear to be derived from a single linear sequence of the HBV DNA genome (see figure 2 ) which is designated the S + pre-S + pre-S1 gene (Tiollais and Wain-Hobson, 1984.). The two major proteins are the product of the S gene, the larger of which is glycosylated. (Peterson, 1984).

### Alpha-foetoprotein (AFP).

Alpha-foetoprotein is a type of serum protein usually found in foetal serum and also in extremely small quantities in the serum of a normal adult (less than 10 ng/ml). Raised serum concentrations of AFP :-up to 500 ng/ml, are seen in 30-40% of patients with acute viral hepatitis. Highest concentrations, up to 5000 ng/ml, are seen in some cases of fulminant hepatitis. In 1963 Abelev et al. found AFP in the blood of a mouse in which ascites hepatoma had been transplanted. Further, in 1964, Tataniov detected it in the serum of a patient with hepatoma. In Japan the figures showed 77% of hepatoma patients were positive. In the strictest sense, there is no more specificity between AFP and hepatoma than hepatitis and cirrhosis and abnormal pregnancy but in patients with hepatoma, AFP in the serum is detected more frequently and in higher concentrations (10,000 ng/ml or higher) than in other diseases.

### DNA Polymerase.

Kaplan et al. (1973) demonstrated a close correlation between the presence of DNA polymerase and the 42 nm virus (Dane) particle. The presence of DNA polymerase is therefore a useful marker of virus replication. Cappel et al. (1977, 1978) demonstrated that the presence of DNA polymerase activity is often the only marker of hepatitis B virus. HBsAg was detected in 88% of 178 individuals exposed to hepatitis B and another 11% developed core antigen-associated polymerase in the absence of surface antigen. Hepatitis B was later confirmed in almost all of these patients after further serological examination. The presence of HBV DNA polymerase also correlates well with the presence of HBeAg.

HBV polymerase, although originally described as a DNA-dependent DNA polymerase, is now believed to function as a reverse transcriptase in the generation of an RNA intermediate during viral replication and hence serves as a marker of infectivity. The long open reading frame (ORF) of the hepatitis B virus genome (see figure 2) codes for a polypeptide of 93 kb, and has been assumed to be the gene for the viral polymerase. Biochemical assays have been developed for the detection of DNA polymerase (see Zuckerman and Howard 1979) but are not suitable for the mass screening of blood donors.

### Viral DNA.

The presence of viral DNA in the serum can be demonstrated by either the polymerase chain reaction PCR (see page 89) or by DNA hybridisation techniques. Commercial DNA hybridisation will detect picograms of virus material present. (PCR may be considered too sensitive). The presence of viral DNA may be useful as a marker of infectivity, (see page 173) and correlates well with the presence of HBeAg, but in reality virtually all transfused HBV infection has been accounted for by the presence of HBsAg or anti-HBc (Hoofnagle 1990). It is mainly for this reason we do not use viral DNA as a marker of infectivity. Instead we currently use HBeAg and anti-HBe as our infectivity markers (see page 173) since the simple one-step RIA in current use (see appendix) is free of charge to us and is easy to perform, with extremely clean results.

### Host Response.

#### HBsAg.

HBsAg, including the antigenic products of the pre-S region, is an early marker of HBV infection. Production of antibodies to HBsAg may be delayed but antibodies to pre-S antigens may occur quite early in infection.

The discovery of the "Australia antigen", now known as the surface antigen (HBsAg), and its association with hepatitis

(Prince, 1968; Blumberg et al. 1968 ) was an enormous step forward in the control of the spread of HBV. The bulk of HBsAg is now known to be unassembled viral coat or "surface antigen".

Antibodies to the surface antigen and the core antigen are known as anti-HBs and anti-HBc respectively. HBsAg is shed in excess into the plasma where it may be detected by immuno-assays based on the use of anti-HBs. A third antigen, HBeAg is a soluble antigen, which can be particle associated, and is found only in a proportion of HBsAg-positive sera. The major polypeptide in the core of HBV, p19, carries the antigenic determinants of both HBcAg and HBeAg (Takahashi et al. 1981). A series of inhibition experiments by monoclonal antibodies suggests that HBcAg has a single immunodominant epitope (Waters et al. 1986). The DNA sequence of HBV is known and part of it corresponds to the amino acid structure of HBsAg (Tiollais et al. 1981).

HBV provides an excellent model for transmission by blood transfusion. In addition to its stability, many of the infections in adults are subclinical. Because of the length of the incubation period, (around three months), there can be a prolonged symptomless viraemia which, coupled with the carrier state, has made HBV until recently one of the most frequent clinical infections transmitted by untested blood and blood products.



### Anti-HBs.

Most individuals who recover from hepatitis B infections develop anti-HBs (Tedder. 1980); the presence of anti-HBs in the plasma protects against re-infection with the same strain of virus. (Zuckerman and Howard 1979). Recipients of multiple transfusions, such as haemophiliacs, have a high frequency of anti-HBs due to the high rate of transfusion associated hepatitis (TAH) found in these groups following the transfusion of clotting factor concentrates prepared from large pools of plasma. (Mollison, Engelfriet and Contreras 1987)

### Anti-HBc.

Although normally preceded by detectable HBsAg at the onset of acute infection, anti-HBc can be demonstrated in most persons who have been infected with HBV. It may be first detected during the incubation period, sometimes before, but generally following the appearance of HBsAg (see figure 5), and persists thereafter. High levels of IgM anti-HBc have been found in persons undergoing acute infection and have been shown to persist for 3-4 months, with replacement by IgG anti-HBc. Raised levels of IgM have been observed in some carriers, although lower than in acute infection.

Anti-HBc IgM is always present in the serum of HBV carriers during the immunoelimination of HBV infected cells during both acute and chronic hepatitis B. (Brunetto et.al. 1991).

Most commercial assays (including our reference test) were designed to detect only high levels of anti-HBc IgM in patients with acute HBV. Low levels of this antibody are specific markers of HBV-induced liver disease when they persist in the carrier, therefore a quantitative determination is necessary.

In carriers, high titres of IgG anti-HBc may be detected. During the recovery phase of an acute hepatitis B infection, anti-HBc may be present in the absence of HBsAg and anti-HBs. Donations taken at this time may sometimes be implicated in transmission of post-transfusion hepatitis (PTH) (Hoofnagle et al. 1978). In a small proportion of carriers the only serological HBV marker present is anti-HBc; some of these individuals may transmit HBV by transfusion.

Anti-HBc is present in virtually all patients with chronic hepatitis B, and virtually all acute HBsAg cases. Why then is not anti-HBc used to diagnose hepatitis B? This is because anti-HBc is also present during convalescence and long after recovery from HBV infection. To screen blood donors for anti-HBc in the absence of HBsAg or anti-HBs has been proposed as a means of prevention of the few cases of hepatitis B (1%) that occur in recipients of blood transfusions despite testing all donor blood for HBsAg. This proposal is based on retrospective studies suggesting that this serological profile is associated with the transmission of hepatitis B to recipients of their blood (Hoofnagle et al., 1978). At present however the test is not generally

used because there is a need to screen all anti-HBc positives for anti-HBs, since this is an indication of immunity (Johnson and McFarlane 1990.). There is a significant false-positive rate and care must be taken when assessing the significance of samples weakly reactive for anti-HBc, especially in the absence of other HBV markers (Tedder, Cameron, Wilson-Croome et al. 1980).

Anti-HBc testing is costly and should not replace HBsAg testing as this is normally produced earlier in the course of infection. In America the test is used as a surrogate marker for non-A, non-B hepatitis in conjunction with ALT levels.

#### HBeAg and anti-HBe.

HBeAg is associated with the presence of large numbers of infective virus particles and hence is a marker of infectivity (see page 37). It was first described in 1972 by Magnus and Espmark. Despite extensive studies since that time, the relationship between HBeAg and the structure of HBV remains unclear. It differs from HBsAg and HBcAg in that it is a soluble protein, not virus-bound. It is associated with high titres of virus and serological markers of hepatitis B virions. Evidence indicates that HBeAg correlates with active underlying liver disease. HBeAg is encoded by the core gene, possibly with a different promoter site, and so is associated with virus replication. It is found during acute infection within the period of HBsAg positivity (see figure 5). During recovery anti-HBe

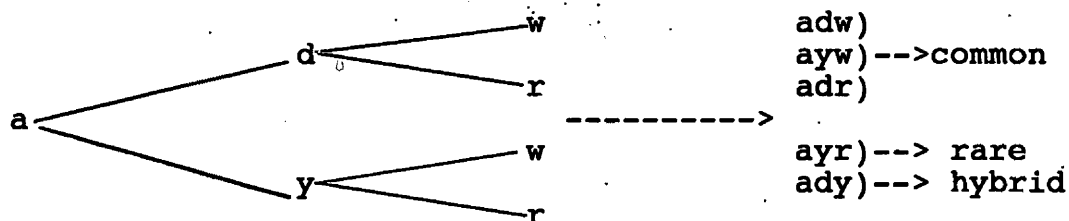
develops. Some chronic carriers who have seroconverted are still undergoing active viral replication due to mutation in the core gene (see pages 46-47 ). Active viral replication is associated with a rise in serum ALT levels due to breakdown and damage to the infected hepatocytes (see page 172). Carriers have HBeAg in their blood initially alongside a high level of HBsAg. This phase may last for a variable period of time. In the U.K., approximately 20% of HBsAg-positive blood donors have HBeAg, (Dow et al. 1980; Harrison et al. 1985) compared with one report of 50% in Mediterranean countries (Lieberman et al. 1983). Anti-HBe eventually replaces HBeAg with an accompanying fall in HBsAg levels. Although the risk of transmission of the virus is greatly reduced during this second phase, transfusion is still very likely to result in HBV infection. Patients who are chronically positive for HBsAg can be divided into three groups: (A) HBeAg positive, viraemic, and with chronic liver disease; (B) HBeAg negative, anti-HBe positive , yet viraemic and frequently with more severe and rapidly progressive liver disease; and (C) anti-HBe positive without viraemia or liver disease. This last group are either from group A and have seroconverted or have gone from acute hepatitis into a simple carrier state without chronic hepatitis developing. From the second group the possibility arises that HBeAg may modify the course of the disease.

### 1:5 Subtypes of HBV.

Levene and Blumberg first recognised in 1969 that HBsAg exhibits antigenic heterogeneity. Le Bouvier in 1971 described three major antigenic determinants. All HBV strains have one antigenic determinant in common, designated a, which may be of subtype d or y, the resulting subtypes being ad, ay, and in some cases ady (Barbara et al. 1977).

Bancroft in 1972 described additional allelic determinants which may be either w or r, which are independent of d and y. Thus the main antigenic subtypes of HBsAg may be summarised in simplified form as follows:

Figure 6.



The view presented above is somewhat oversimplified because subunits of a as well as a large number of minor antigenic subdeterminants have been described.

Figure 7.

Subtype determinants of HBsAg.

group reactive	<u>a</u>
allelic pairs	<u>d</u> <u>y</u>
	<u>w</u> <u>r</u>
primary subtypes	<u>adw</u> , <u>ayw</u> , <u>adr</u> , <u>ayr</u>
additional determinants	x, n, j, t, k, q,

Determination of subtypes is useful epidemiologically, since their distribution varies geographically and between sections of the community. For example the ay subtype is predominant among donors from high hepatitis risk regions such as the Middle East and the Mediterranean, but second generation immigrants tend to adopt the pattern of the host country. Homosexual HBsAg carriers in the U.K. are usually of the ad subtype, whilst drug addicts are of the ay subtype (Barbara, 1983). Subtype remains the same during acute and/or chronic infection in an individual and breeds true when transmitted to a susceptible host. Subtype antigen elicits antibody to the determinants of that specific subtype.

### 1:6 Variants of HBV.

During immunisation programmes with approved vaccines on contacts of hepatitis B in southern Italy , some of those vaccinated have shown signs of infection with a variant mutation of the hepatitis B virus, despite production of detectable antibody. (Carman et al. 1990)

Between 1982 and 1987 infants and other contacts of HBV carriers from two regions of Southern Italy were vaccinated by both passive and active vaccines. Their immune response was monitored and in one group of 1,590, 44 (2.8%) contacts of HBV carriers became HBsAg positive despite the presence of vaccine-induced antibody. Severe infection occurred in one infant who progressed to the carrier state. Samples of the child's DNA revealed that a point mutation from guanosine to adenosine at nucleotide position 587 resulted in the substitution of arginine for glycine in the highly antigenic 'a' determinant of HBsAg, to which a large part of the vaccine-induced immunity is directed. This mutation was stable as it was present in an isolate from the child five years later. The mother's virus was different from that of the child, and it is thought that the mutation occurred during infection. In this child and in some of the other patients the group-specific 'a' determinant was partially missing. This must be kept in mind for the design of future vaccines since most vaccines are directed towards the 'a' determinant. Hence any mutation in this area may result in an "escape mutant" virus which evades.

vaccine-induced immunity, although patients in which the variant virus has been found were all vaccinated using plasma-derived vaccines.

A viral infection characterised by the presence of serum HBsAg positivity without serum anti-HBc has been encountered in Senegal. It is now known that the virus implicated in this infection has an identical DNA structure to that of HBV and is the result of a different response in certain individuals, after HBsAg positivity is lost neither anti-HBc nor anti-HBs become detectable. This is considered to be a lack of adequate antibody response in the individual rather than a genuine case of virus variation although the reasons for an apparent "clustering" of cases are not explained.

Point mutations in viral sequences have important effects on pathogenicity. Further mutations of this kind have been observed in a study carried out in London and Athens on 18 Greek and 3 non-Greek patients positive for HBsAg but negative for HBeAg (Carman et al. 1989)

A further study by Kojima et al. 1991 described fulminant hepatitis B associated with precore-defective HBV mutants. Direct sequencing of HBV DNA amplified from sera showed mutations in 7 out of 8 HBeAg negative patients. Two mutations (guanosine to adenosine) were found in the terminal two codons of the pre-core region. The remaining patient had the first of these two mutations only. It is thought that changes in the nucleotide sequence of the



pre-core region of HBV are responsible for the failure of some patients with continuing viral replication and severe liver disease to secrete HBeAg. The first mutation results in a translational stop codon at the end of the pre-core region that will prevent HBeAg production, because HBeAg is a peptide derived by continuous translation from the pre-core region through into the core region. The second mutation results in a stop codon which may reduce the efficient translation of core antigen.

At present the existence of mutant or defective forms of the virus does not present a major problem in the context of transfusion microbiology in this country as these forms appear so far very rare. There is only a problem for microbiological screening if the coating antibody used on the solid phase is not sufficiently pan-specific to detect all mutant or variant forms of HBsAg (see section 3 on assays used to detect HBsAg). However consideration may have to be given to the inclusion of mutant sequences in future vaccines to prevent emergence of escape mutations.

### 1:7 Chronic Carriage of HBsAg.

The term "carrier" in general medical usage indicates an infected individual who is able to transmit an agent but who lacks manifestations of the disease. In the case of HBV however the wide spectrum of hepatic manifestations of HBV infection range from normal liver to severe chronic active hepatitis and primary hepatocellular carcinoma (PHC). The carrier state is more common in males, more likely to follow infections acquired in childhood than those acquired in adult life, and more likely to occur in patients with natural or acquired immune deficiencies. (Zuckerman and Howard 1979).

The differentiation between an acute infection and the carrier state is of great importance both clinically and epidemiologically. A true carrier state for the hepatitis B virus has been confirmed by a series of epidemiological and clinical findings made by a number of workers, including the evaluation of immunopathology observed in liver biopsy specimens. The reasons for becoming a carrier are unknown but it probably represents a selective failure of the host immune system (Tedder. 1980). Although carriers are not generally immunoincompetent, infections occurring in immunosuppressed persons are known to be more likely to progress to a carrier state. The high rate of carriage in certain populations, e.g. China and Asia may be explained by early infection when the immune system is less competent. There also appears to be a genetic

predisposition towards the carrier state as shown by its association with Down's Syndrome.

The individual who exhibits the least symptoms is most at risk of becoming a carrier, since he is unable to mount a strong immune response to the virus. Most adult infections are symptomatic and less likely to become carriers (approx. 5%). Most adult infections are associated with some form of risk behaviour.

The present study is only concerned with investigations into some serological aspects of the carrier state.

Evidence that a prolonged carrier state may result in some patients has been provided by Stokes et al. (1954), Neefe et al. (1954) and Murray et al. (1955a,b). Zuckerman and Taylor (1969) demonstrated a well-documented case of apparently healthy carriage of hepatitis B antigen for over 20 years. Giles et al. (1969) tested for HBsAg in serial samples of serum obtained before and up to 200 days after experimental infection of volunteer children with serum containing the MS-2 strain of type B hepatitis. HBsAg was detected in all patients after infection with MS-2, often before there was any clinical evidence of infection. In approximately half of the patients the antigen persisted in the serum for 200 days and subsequently for at least 3 years after infection. With the development of sensitive assays for the detection of HBV markers, it has been estimated that there are some 2 to 3 hundred million carriers in the world (Lever, 1987).

The WHO Expert Committee on Viral Hepatitis, 1977 defines the persistent carrier state as the presence of hepatitis B surface antigen for 6 months or longer. The hepatocytes of the carrier are chronically infected with HBV and are directed by the viral genome to produce excess HBsAg. As a result of this chronic infection the carrier circulates HBsAg and may manifest a variety of other serological markers reflecting the presence of the virus (see earlier section on HBV markers). The carrier state is not static, the changes which occur with time are similar to the changes seen in acute hepatitis B, but over a much longer period of time.

Extensive epidemiological surveys have shown that the prevalence of HBsAg in apparently healthy persons varies from 0.1% to 20%. Europe may be roughly divided into three regions (i) northern countries, with a low prevalence of about 0.1% or less (ii) central and eastern countries, with a 0.1% to 3% prevalence, and (iii) countries bordering the Mediterranean, where there is a prevalence of about 5% or even higher. The prevalence in North America and Australia is 0.1% or less, 3%-5% or higher in Asia, as high as 15 % in several tropical countries and as high as 20% in some areas, particularly in the Far East, with evidence of presence of HBsAg and/or anti-HBs in almost the whole population. However, in examining the global distribution of HBsAg, it should be noted that standardised tests and reagents have not been employed and that full distribution has not as yet been completely documented.

In 1978 Drew et al. put forward the hypothesis that there is cross-reactivity between the surface antigen and a male-associated antigen. If this is the case, males would be more likely to recognise the surface antigen as "self" and therefore would persistently carry the antigen. Females however, would be more likely to recognise the surface antigen as "foreign" and therefore produce surface antibody. This may account for the excess of male carriers noted by most researchers in this field.

In 1969 Blumberg put forward a genetic hypothesis to explain the geographical variation of apparently healthy carriers of surface antigen. As a result of family studies carried out in Cebu and Bougainville in the Pacific, he hypothesised that a gene designated "Au<sup>1</sup>" is responsible for persistence of the surface antigen after infection with hepatitis B virus has occurred. This gene is considered to be common in tropical areas and rare in temperate zones.

In countries in which infection with hepatitis B virus is relatively uncommon, the highest prevalence is found in the 20-40 year age group, whereas in those countries where infection with hepatitis B virus is relatively common, the highest prevalence of HBsAg is observed in children 4-8 years old, with declining rates among older age groups. The decline in antigen carriage rates with age suggests that the carrier state is not inevitably life-long. The prevalence of surface antibody increases with age. Hepatitis B e antigen is found more commonly in young

rather than in adult carriers while the prevalence of anti-e appears to increase with age (Ohbayashi et al., 1976). These findings suggest that young carriers may be the most infective.

Carriers may be divided broadly into two categories of infectivity, depending upon the presence in the serum of the HBe antigen or its antibody. About 1 in 4 U.K. carriers have HBeAg in their serum. It is likely that such persons will have recently become carriers and that their blood will be highly infectious. More commonly, the carrier will have anti-HBe in his or her serum and will be at a later stage of the infection. Although anti-HBe carriers are far less infectious, the majority are still likely to transmit infection by transfusion. For this reason all HBsAg-positive donors must be excluded from giving blood for transfusion purposes.

With a volunteer donor population, the most reliable method of preventing HBV transmission is the screening of blood donors for the presence of HBsAg. It is known that a donor can transmit HBV as long as 19 years after having had a positive HBsAg test (Zuckerman and Taylor, 1969). It has been estimated that carriers clear their HBsAg at a rate of about 1.7% per annum (Sampliner et al. 1979; Barbara, 1983). If and when carriers eventually lose their HBsAg, it is usually succeeded by detectable anti-HBs.

More than 70% of apparently healthy British blood donors found to be HBsAg-positive gave normal liver function test

results, as indicated by serum aspartate transaminase (AST) and alanine transaminase (ALT) levels, and were associated with anti-HBe positivity. (Barbara et al. 1978). In Dutch studies a similar figure was observed. In the remaining 30%, when the tests remained abnormal over a period of time, moderate to severe liver disease was present at biopsy in 8 out of 9 cases. Abnormal liver function tests (LFTs) (see section 4 for table of normal values) were found significantly more often in HBeAg-positive carriers than in anti-HBe-positive carriers (Reesink et al. 1980).

### Liver Disease in Carriers.

Various surveys have found a proportion of asymptomatic carriers of HBsAg to have abnormalities of the liver. These abnormalities range in severity from minor changes in the nucleus of the cell to severe chronic active hepatitis and cirrhosis. The subject has been extensively reviewed by Zuckerman (1988) and others. Primary hepatocellular cancer is rare in the U.K. Carriers are more at risk of developing chronic active hepatitis and cirrhosis. These studies suggest that asymptomatic carriers should remain under clinical and serological observation to reduce these risks, alteration of lifestyle and if necessary medical or surgical intervention may be appropriate.

### The Link Between HBV and Primary Hepatocellular Carcinoma.

In the Far East and in sub-Saharan Africa, chronic hepatitis B infection is frequently associated with the development of human hepatocellular carcinoma (HCC). This association between HBV and HCC makes it one of the most frequent fatal malignancies worldwide. Recent estimates (Zuckerman, 1983) range from 250,000 to 1,000,000 deaths per year from HBV-associated HCC. It is to be hoped that HBV vaccination will eventually help to eliminate this common cancer of mankind.



There are many laboratory assays currently on the market for the detection of HBsAg in donor blood. The next section shows how we screen blood for this marker of HBV infection.

**SECTION 2: THE PRACTICE OF TRANSFUSION MICROBIOLOGY AND A**

**DESCRIPTION OF THE ASSAYS INVOLVED.**

## 2:1 Microbiology in Blood Transfusion.

The National Blood Transfusion service was first formed in 1946 under the provisions of the National Health Service Act. The service in the U.K. overall, including the Scottish National Blood Transfusion Service now comprises 19 Regional Transfusion Centres, a Blood Group Reference Laboratory and three Blood Products Laboratories.

The North London Blood Transfusion Centre located at Colindale North London where this study was prepared, supplies specific requirements for many of the London teaching hospitals, most of the blood used by general hospitals in the region as well as a large percentage of the plasma supplied to the Blood Products Laboratories from which preparations such as factor VIII and immunoglobulin preparations are manufactured

Blood transfusion has progressed sufficiently since the first transfusion by James Blundell in 1818, to allow the use of component therapy which means that a single donation can be split into components such as concentrated red cells, washed red cells, frozen red cells, platelets, dried plasma and plasma protein fractions and hyperimmunoglobulin, and used to treat several patients.

Conrad(1981) stated that the transmission of infection is the most frequent serious complication of transfusion therapy. Microbiological screening is therefore a necessity

in the provision of a safer, low-risk blood supply and in the avoidance of some of the unwanted side effects of transfusion.

Many of the requirements for microbiological assays in the transfusion service are common to other disciplines, but transfusion microbiology makes its own special demands. Testing must be rapid, to ensure that urgent requests for blood can be met at the earliest possible opportunity, but must also be of a high degree of sensitivity and applicable to hundreds of samples daily. Obviously when large numbers of samples are being screened, cost is important, but there is also a need for low false-positive rates and simple methods using inexpensive and easily maintained equipment.

In addition we need a test which is specific enough not to alarm the donor unnecessarily or to waste his valuable unit of blood through a mis-diagnosis.

Meeting these requirements may appear impossible but the methods employed in the detection of hepatitis B surface antigen (HBsAg) in donor blood stand as a model for rapid, mass viral diagnosis.

## 2:2 The role of automation in transfusion microbiology.

As the list of mandatory screening tests and the requirements for rapid release of blood increases, the chances for making laboratory and clerical errors also increases. Chambers et al. reported a 0.3% error rate in the throughput of a large biochemistry laboratory. The advent of formal product liability highlights the obligation to make the blood supply as safe as possible. One obvious approach is to provide accurate information flow and to expand and develop the use of automated systems in the laboratory.

The prime target must be the automation of sampling to provide specific sample identification with direct input to the main computer system. Ideally this should be linked with information transfer from the readers at the various assay stations to minimise or eliminate manual data flow. Results and sample identity can then be centrally integrated prior to release of blood or components.

Other aspects of laboratory testing may also be automated as appropriate. All screen tests involve several critical laboratory steps as follows:

- (i) addition of correct sample to specified well
- (ii) addition of correct reagent
- (iii) procedural steps (various)
- (iv) recognition and flagging of reactive samples
- (v) correct identification and retrieval of samples

- (vi) appropriate "hold" and donation/component quarantine
- (vii) validation and confirmation of reactivity
- (viii) re-testing a follow-up sample from the donor.

As the numbers of tests performed increase, so the chances of error also increase. Deferrals of donors and/or donations may be permanent or temporary, thus adding to the complexity of information flow. Samples giving false-positive reactions, as well as those giving positive reactions, must be temporarily deferred pending follow-up. An added complication is the need to provide a panel of male volunteer donors who are "accredited" as having a record of documented "safety", in order that they may be boosted with anti-rhesus antibodies to provide a vaccine to immunise rhesus-negative mothers against producing antibodies to rhesus-positive babies. A variety of test results (often non-standard) have to be integrated with follow-up testing and/or quarantine, and these data must be co-ordinated.

In order to reduce errors of information transcription, laboratories should aim to minimise manual data transfer, employ systems based on positive sample identification and move towards the computerisation of recording results of tests analysing these results and transferring the data.

Total automation of testing may be impractical where different methodologies are employed. At NLBTC we process up to 1000 samples, totalling over 4000 tests daily. The use of the microtitration system wherever possible and the

careful use of modern automated samplers can greatly increase the rate of throughput. At NLBTC we favour the use of sub-sampling from a 96 well microplate filled by an automated sampler, because, although automation gives precision of information transfer, it can actually take longer than manual sampling where multiple tests are performed on any one sample. In centres such as ours, with a large throughput of samples and a wide range of tests, one may compromise by making a small number of masterplates on an automated sampler and then sub-sampling, thereby maintaining most of the benefits of automation but achieving the best compromise as regards speed of operation. Various machines (e.g. the Costar Transplate) can then remove 96 samples at a time in appropriate volumes for a variety of simultaneous screen tests. Direct input from ELISA readers, RIA gamma counters or HA image analysers into the main computer can then integrate with the sample-position data fed in from the automated sampler.

### 2:3 Summary of routine practice in HBsAg testing at NLBTC

(For an illustration see figure 8)

Serum samples are supplied for microbiological assays by the staff at the donation sessions. Each donation carries its own unique identification number. One serum tube is supplied per blood unit collected. The caps are removed from the tubes manually and all tubes are centrifuged to leave 1.5 cm. of clear serum standing above the blood clot. The sample tubes are then placed in aluminium sample "blocks" in numerical order and the location of each sample is identified on the record sheets generated with the samples at the donor sessions.

The sample tubes are then sub-sampled for the various tests performed in the laboratory. For HBsAg testing, 0.1 ml of serum is placed simultaneously, using the Costar Transplate, into the wells of a commercial EIA assay plate coated with guinea-pig anti-HBs. A suitable range of positive and negative control sera are included on each plate and the plate is incubated for 20 minutes at 40°C on a shaker incubator. The plates are then washed on a plate washer and 0.1 ml of goat anti-HBs conjugated with peroxidase is added to each well of the plate using a multi-channel pipette. The plate is then re-incubated on the shaker for a further 20 minutes. The plates are then re-washed and 0.1 ml of substrate containing hydrogen peroxide and TMB chromogen is added to all wells of the



plate. The plate is allowed to incubate on the bench at room temperature for 10 minutes whilst a blue colour develops in those wells where HBsAg is present. The reaction is stopped by the addition of sulphuric acid which turns the blue colour to yellow. The resulting colour is read spectrophotometrically. Any samples giving an initially positive reaction are re-tested in duplicate.

An attempt is made to specifically neutralise a repeatably reactive sample with an anti-serum containing specific HBsAg antibody in order to confirm conclusively any positive reaction (see appendix 4.). A positive neutralisation test indicates the presence of HBsAg in the sample. Failure to neutralise with specific antibody would suggest a false positive reaction and the sample is sent to the reference laboratory for tests for all HBV markers to confirm this.

BATCH NUMBER... = D  
Cut-off absorbance = 0.048  
Low control limit = 0.011  
High control limit = 0.034

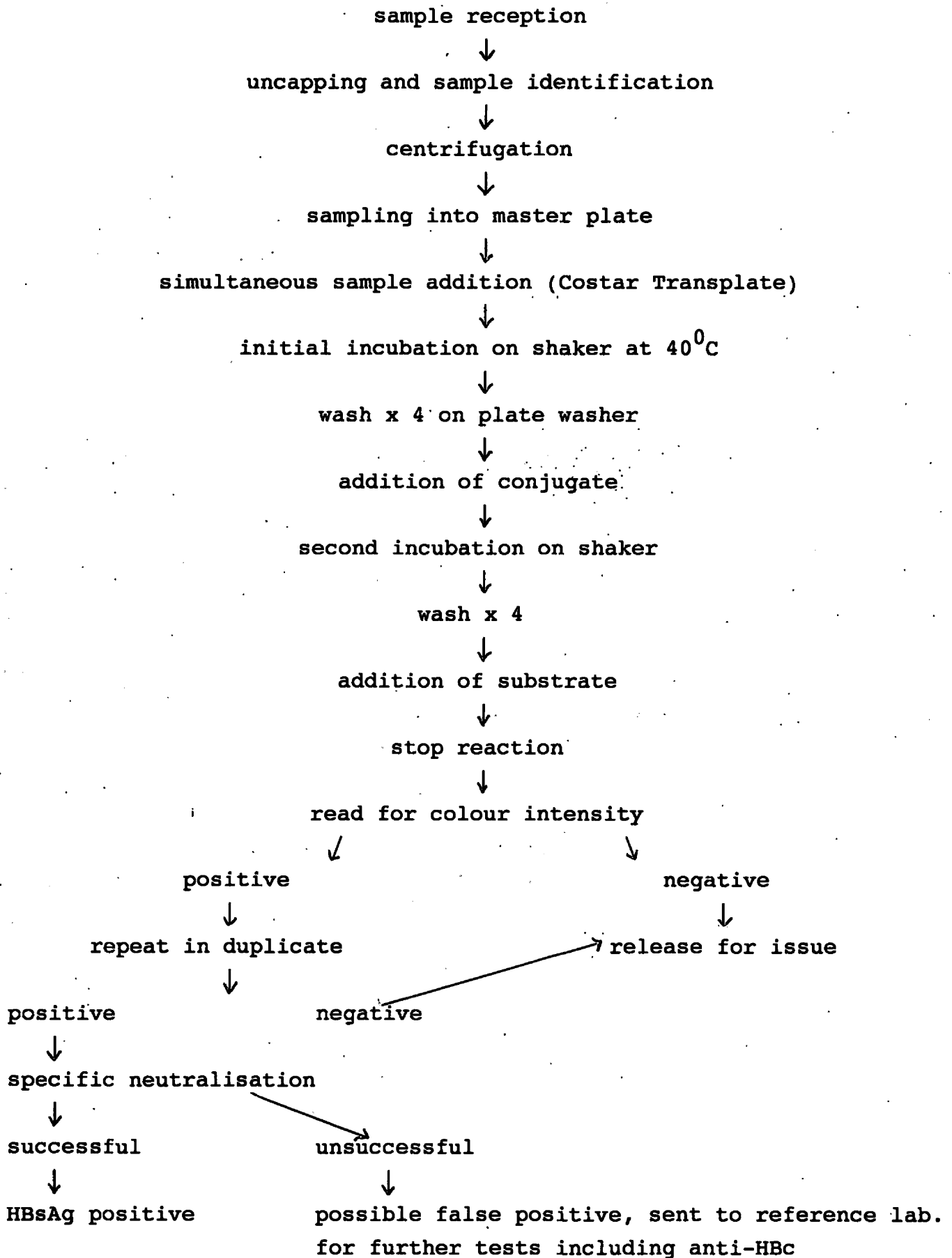
NEG =	0.017	0.022	0.021	0.022	0.031
POS =	0.543				

Plate number : 1  
Variables : ABSORB RESULT

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.017 NEG	0.021 NEG	0.031 NEG	0.055 !!POS!	0.024 ....	0.021 ....	0.022 ....	0.017 ....	0.018 ....	0.029 ....	0.018 ....	0.026 ....
B	0.022 NEG	0.022 NEG	0.543 POS	0.137 !!POS!	0.023 ....	0.024 ....	0.025 ....	0.028 ....	0.031 ....	0.025 ....	0.026 ....	0.024 ....
C	0.017 ....	0.012 ....	0.016 ....	0.016 ....	0.020 ....	0.017 ....	0.016 ....	0.015 ....	0.025 ....	0.017 ....	0.015 ....	0.023 ....
D	0.022 ....	0.017 ....	0.021 ....	0.017 ....	0.019 ....	0.019 ....	0.018 ....	0.022 ....	0.019 ....	0.019 ....	0.015 ....	0.014 ....
E	0.019 ....	0.019 ....	0.024 ....	0.020 ....	0.032 ....	0.021 ....	0.024 ....	0.020 ....	0.024 ....	0.020 ....	0.016 ....	0.017 ....
F	0.021 ....	0.025 ....	0.022 ....	0.024 ....	0.023 ....	0.020 ....	0.023 ....	0.025 ....	0.019 ....	0.018 ....	0.013 ....	0.013 ....
G	0.009 ....	0.009 ....	0.010 ....	0.011 ....	0.014 ....	0.014 ....	0.016 ....	0.014 ....	0.015 ....	0.016 ....	0.011 ....	0.016 ....
H	0.020 ....	0.023 ....	0.022 ....	0.021 ....	0.020 ....	0.020 ....	0.017 ....	0.020 ....	0.017 ....	0.019 ....	0.017 ....	0.018 ....

**Figure 9.**  
Specimen print-out of HBsAg screening results at NLBTC by the Bioelisa EIA assay. The negative controls which determine the cut-off O.D. are in positions A.1., A.2, A.3, B.1 and B.2. The kit positive control is at position B.3 and the BPL 0.25 ng/ml standard is in A.4 and the 0.5 ng/ml standard is in B.4. The rest of the plate consists of negative donor sera.

Figure 8. Flow-chart to illustrate HBsAg testing procedure at NLBTC.



Standard procedure for validation of HBsAg positive results.

When a sample has been identified as HBsAg positive by routine screening it is then tested in duplicate and a sample from the actual pack is obtained and tested to exclude any clerical or labelling errors or cross-contamination of the sample. Confirmation of the specificity of a positive reaction is essential before the donation can be reported as HBsAg positive. Incorrect positive reports give rise not only to false information in epidemiological studies (Communicable Disease Reports) but also to a variety of unnecessary difficulties for donors wrongfully designated as HBsAg positive. The positivity is therefore validated and confirmed by specific neutralisation (see appendix 4.) However, since we have changed from SPRIA to EIA, validation is not so clear-cut because EIA assays are more prone to the problems of false positivity.

The sample is then examined for supplementary tests, some of which can be performed in-house but most of which are done at the reference laboratory. These supplementary tests consist of anti-HBc, both IgM and total, HBe antigen and antibody, surface antigen (and also antibody in the case of known previous infections). By performing these supplementary tests we are able to see in what phase of infection the donor currently is, and by repeatedly sampling from the donor at regular intervals we are able to

monitor the progress of the infection and the donor's likely infectivity status.

Categorisation of HBsAg positive donors.

1. Anti-HBc negative, HBsAg positive - very early acute infection.
2. Anti-HBc positive, anti-HBc IgM positive, HBsAg positive - some phase of acute infection( up to 6 months).
3. Anti-HBc positive, Anti-HBc IgM negative HBsAg positive - chronic carrier.
4. HBsAg negative, anti HBc positive anti-HBs positive - convalescent/immune.

Within either an acute or chronic infection, the donor could be either HBe antigen or antibody positive. In an acute infection anti-HBe associates with resolution of the infection. In chronic carriage, HBeAg associates with high infectivity while anti-HBe associates with lower infectivity.

When the results of all these tests are known, the donor is counselled and a confirmatory sample is obtained. This confirmatory sample is titrated in parallel with the original sample. This serves to show that there has been no mistake as to the identity of the donor and also to provide "kinetic data" i.e. to look for significant changes in the

titre of HBsAg. If on titration of the confirmatory sample the donor appears to be HBsAg negative the results of an anti-HBc test (including an anti-HBc IgM) and an anti-HBs test will give significant information about his status.

## 2:4 Immunoassays in Transfusion Microbiology.

### Evolution of HBsAg testing.

Modern technology has contributed enormously to the production of diagnostic assays over the last two decades, especially since the development of monoclonal antibodies and recombinant or synthetic polypeptides. These have led to increasing sensitivity of assays without a disproportionate increase in the number of false-positive reactions. Increased sensitivity has reduced the "diagnostic window" period, between infection and the appearance of detectable markers, helping to make the blood supply safer.

The evolution of HBsAg testing has reflected some of these advances from the early "first generation" assays to the present widely used EIA and other recently evolved serological diagnostic assays, such as chemiluminescence (see page 83).

In addition, there are now available assays which perform direct detection of the viral genome such as the polymerase chain reaction (see later description and figure 11) and also the ligase chain reaction which also makes use of nucleic acid technology. These assays have great potential for the future of viral diagnosis.

### Immunodiffusion.

In the early 1960s Blumberg discovered the hepatitis B surface antigen (HBsAg) in the serum of an aborigine boy (see section 1) using the technique of immunodiffusion. At this point mass-screening of blood for transfusion became possible, and by 1970 had become a reality. The method initially used was that with which HBsAg had first been detected, the simple (but relatively insensitive) immunodiffusion test (ID). This proved effective because of the large amount of excess HBsAg produced by the virus. Since HBV is unable to form plaques in tissue culture or even to be used in 50% end-point assays in animals, virologists have been forced to rely on immunoassays. These have been developed to provide rapid and convenient tests giving minimal false-positive reactions while avoiding the problems that beset cell-culture techniques.

In the technique of immunodiffusion, test serum is introduced into wells cut in an agarose gel. The test serum is then allowed to diffuse outwards to meet a specific antigen or antibody diffusing from another well, an immunoprecipitin line forms at the point where antigen and antibody are at optimal concentrations. Experimental details may be found in a range of publications (e.g. Crowle, 1973).

Though the immunodiffusion test(ID) can be optimised to take 12 hours to perform, dramatic advantages were provided by the introduction of an electric current through the gel:



with this technique, an immunoprecipitin reaction can be made visible in as little as 2 hours . This method known variously as "CIEP" (counter-immuno electrophoresis), "EIOP" (endimmuno-osmophoresis), "CEP" (cross-over electrophoresis), or "CMP" (cross-migration electrophoresis), relies on active movement of charged antigen in one direction and internal osmotic streaming of antibody in the other direction. Since the reactants only move towards each other (and not radially) stopping only at the precipitin line, this results in a rapid and more sensitive assay, with a build-up of precipitin, as the assay does not rely only on diffusion.

#### Reverse passive haemagglutination.

In 1974 commercial RPHA (reverse passive haemagglutination) systems became available. The most widely used in Britain was Hepatest (Cayzer et al., 1974) marketed by Wellcome. This is still used on a routine basis at NLBTC for the follow-up titration of HBsAg positive donors.

Viruses which do not normally produce haemagglutination patterns may be induced to do so by the use of red cells coated with antibody to the virus (see figure 10.). This antibody may be tagged to fresh erythrocytes or to fixed erythrocytes that may then be freeze-dried. The test serum is incubated with the antibody-coated erythrocytes, and after allowing time for settling, the haemagglutination patterns may be read.

The procedure used routinely until March 1991 supplementary to the RIA assay for donor screening at the NLBTC consisted of a modified version of the Serodia-HBs test (Fujirebio Inc.) and has been described by Barbara (1983). In this test the sensitised and unsensitised red blood cells are diluted to 0.1%, instead of the usual 1%, and a "spin and slope" technique replaces the usual method of allowing the cells to settle in the wells. However, during the current study, the original "1%" Hepatest (Wellcome Diagnostics) (Cayzer et al., 1974) was employed for HBsAg quantitation, to allow direct comparability with previous results obtained over a number of years.

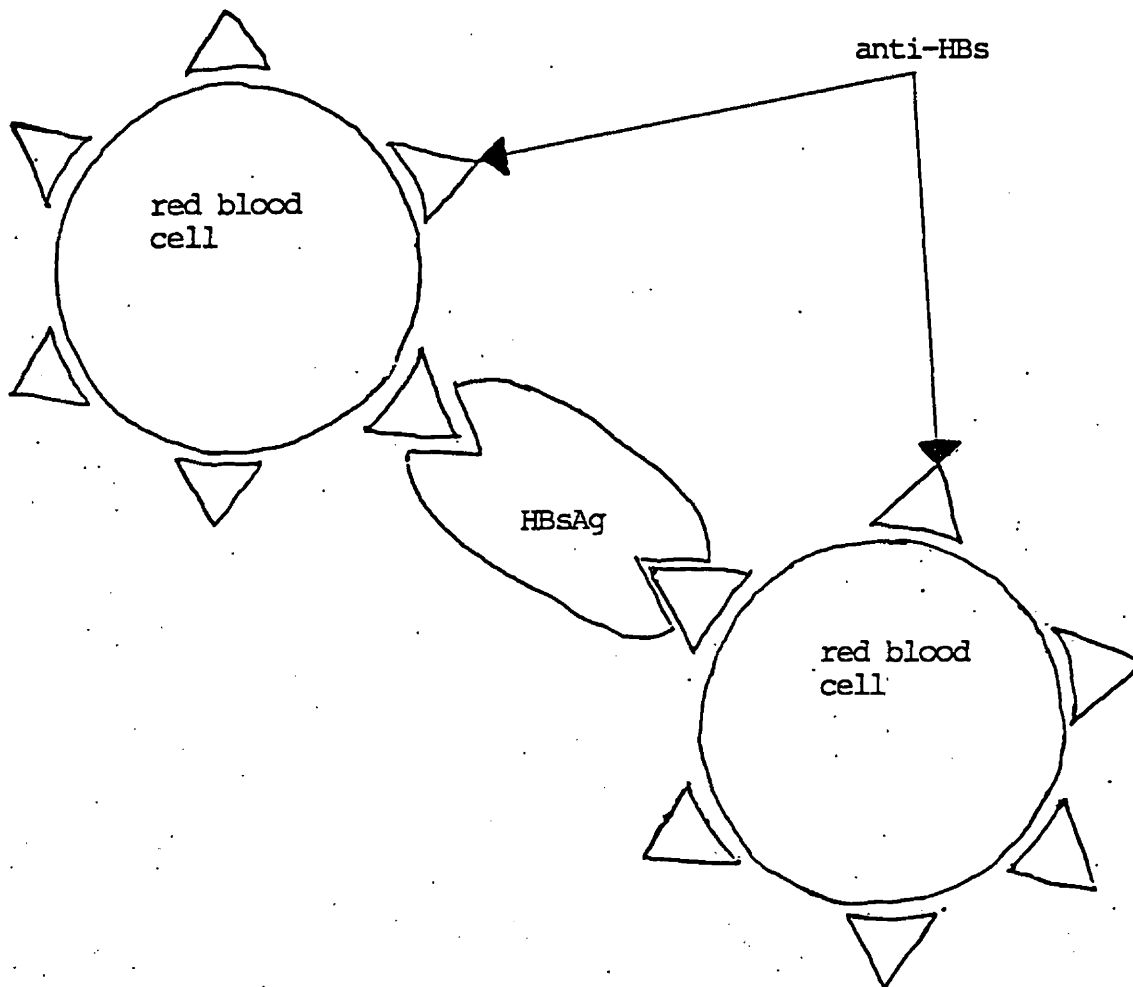
The principle of the test is that red blood cells can be coated with purified antibody. This was originally done by Boyden (1951) using tannic acid, giving the so called "tanned" red cells. These cells can be agglutinated by very small amounts of antigen. Viral agglutination gives rise to a "carpet" of cells rather than the "clumping" seen with bacterial agglutination.

This type of test had an enormous impact on HBsAg testing in the transfusion context, since it lends itself to automation and is performed in the microplate format. RPHA is more expensive than EIOP which can use in-house materials, but is more sensitive and rapid. Use of the test is described in detail in appendix 2. The ability to freeze-dry the cells affords the advantage of prolonged reagent storage. The use of turkey red blood cells (nucleated) in Hepatest allows faster settling and fewer

non-specific cross-reactions with heterophil antibodies than mammalian cells. Serodia-HBs (produced by Fujizoki and marketed in the U.K. by Mast Laboratories) uses chicken red cells in a format similar to Hepatest.

The standard methods of RPHA have been successfully modified to reduce costs and increase sensitivity. An efficient modification that allows the use of diluted cells (and which also increases sensitivity) is to employ the method used by haematologists of centrifuging the test in a "V"-well microtitration plate to sediment the cells after incubation with the diluted test serum and then to incline the plate at an angle to observe if the sediment remains agglutinated or forms a streak (see plate 4.) This method was used at NLBTC using diluted Wellcome Hepatest cells (0.1%), and later using Fujizoki Serodia-HBs cells. The latter method is still used as a quality assurance for donations from previously untested donors.

Classically the red blood cell has been the particle of choice for agglutination assays but recently a Japanese company (Fujirebio Inc.) have developed gelatin particles that mimic the behaviour of erythrocytes in tests requiring agglutination procedures. These are currently available for anti-HIV and anti-HTLV detection. Coated latex particles also offer an agglutination system for antigen detection but are often less sensitive than other immunoassays. ( McDonald and Barbara 1988).



**Figure 10.**

Reverse passive haemagglutination assay (RPHA) for HBsAg. Antibodies to HBsAg are adsorbed on to the surface of tanned red blood cells. Agglutination of these red blood cells occurs in the presence of HBsAg.

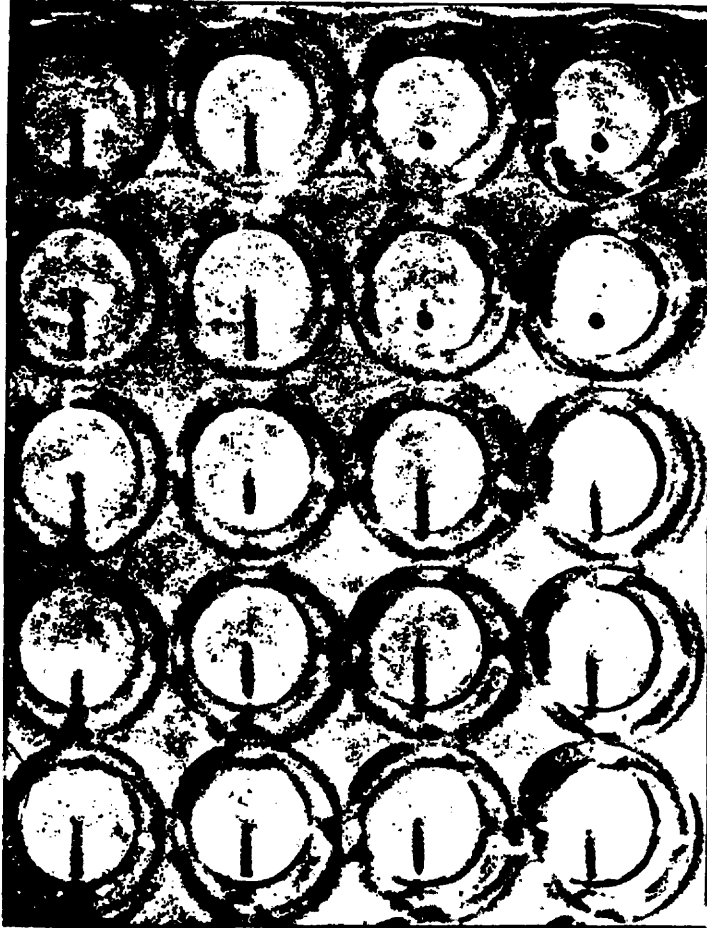


Plate 4. Close-up of the top right hand corner of a microtitration plate showing the four tight buttons of the positive control of the 0.1% modified "spin and slope" HBsAg haemagglutination assay, and the streaking-effect of negative reactions.

### Types of immunoassays.

The more sophisticated assays using plastic microwells, beads or paddles/dipsticks as the solid phase have been classified by Mortimer et al. (1985) as follows (see figure 11 A-E):

1. Type I, antiglobulin
2. Type II, competitive
3. Type III, antibody capture
4. Type IV, sandwich

These four basic formats can each be modified in several ways. A range of systems can then be used to provide "labels" for signalling the specific antigen-antibody reactions. Either antigen or antibody may be labelled. The most commonly used markers for these types of assay are radioactivity, enzymes, lanthanides, chemiluminescence and gold labels. The principles of these types of immunoassay are illustrated in figure 11.

### Radioimmunoassay.

After haemagglutination, the next stage in HBsAg detection was the introduction of the more sensitive RIA assay (radio-immunoassay). The early competitive assays took as long as six days to perform. Radiolabelled antigen was allowed to react with the serum for four days, after which free labelled antigen was separated by chromatoelectrophoresis from labelled antigen bound to specific antibody. After staining, the paper strips on which separation was effected were dried and passed through an automatic strip-scanner for counting. (Walsh, Yalow and Berson 1970). These lengthy and cumbersome assays eventually gave way to the more convenient solid phase "sandwich method" routinely employed until recently at NLBTC and described in appendix 1. Linking antibodies to particles or surfaces gives a solid-phase reagent. Early commercial tests were performed in coated tubes although coated beads later became popular. RIA was used at NLBTC until March 1991 when an EIA assay was introduced for HBsAg testing of all donations.

The principle of the assay for HBsAg is the adsorption of viral antibodies on to a solid matrix surface. Various materials may be used, for example, disposable plastic tubes, plates or beads, made in polystyrene, polypropylene or polyvinylchloride. The antibodies immobilised at the solid phase provide binding sites for viral antigens in the test serum which is subsequently added to these bound

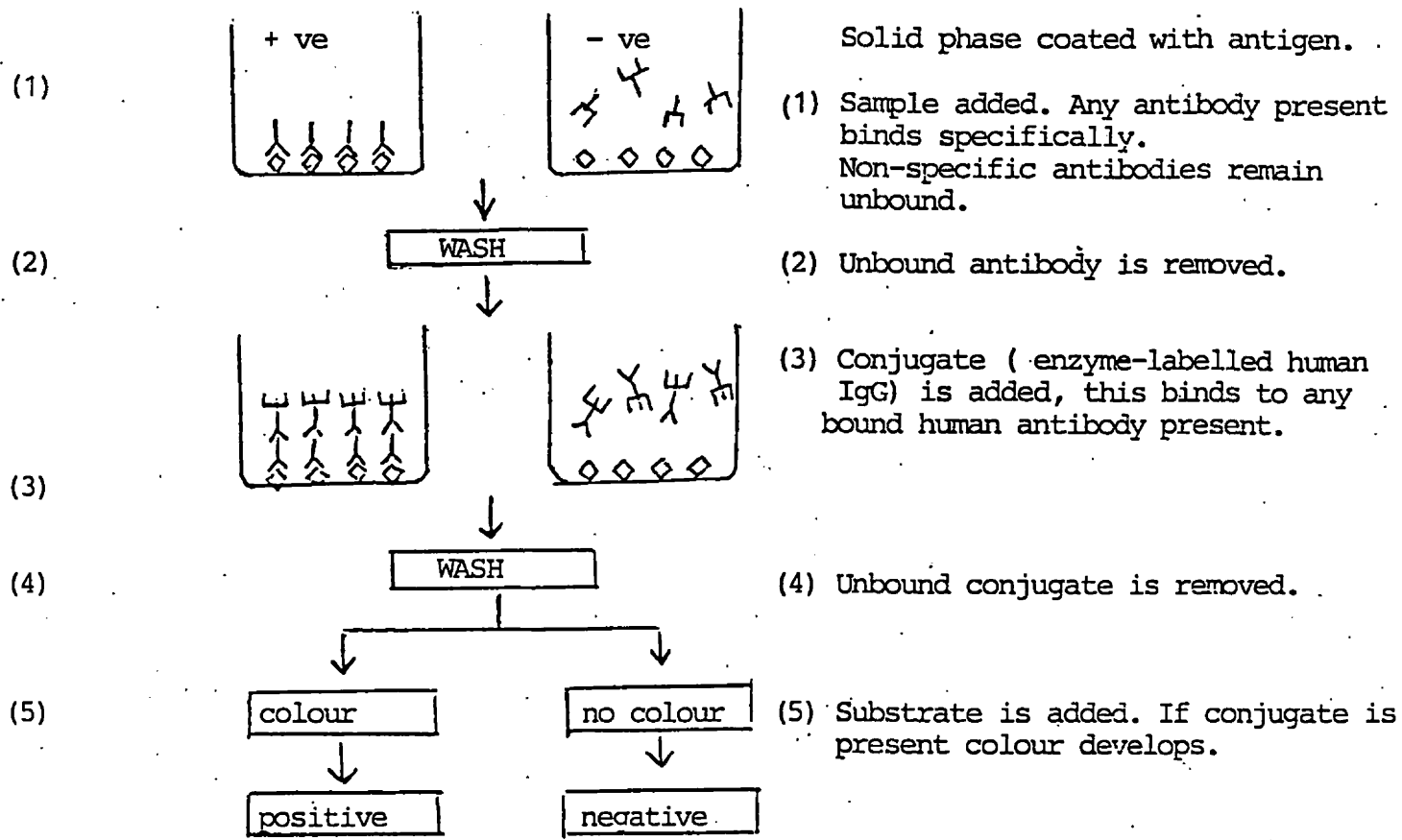
antibodies. The bound antigen is then detected by the addition of  $^{125}\text{I}$ -labelled antiviral immunoglobulin G (IgG). This then forms an antibody-antigen-antibody "sandwich" attached to the solid phase. The amount of bound  $^{125}\text{I}$ -labelled antibody is measured with a gamma counter and expressed as a ratio of counts-per-minute compared with a set of appropriate controls.

The technique used in this study has been described by Barbara et al. (1979a) and Cameron et al. (1980) and was used routinely in the screening of blood and blood products at the NLBTC until March 1991. The microtitre format is employed (Barbara 1983) in which strips of light, small polystyrene wells ("Remova-strip", Dynatech Laboratories Ltd.) are fitted into a polystyrene tray (Dynatech Laboratories Ltd.) to form a 96-well, microtitre-format plate.

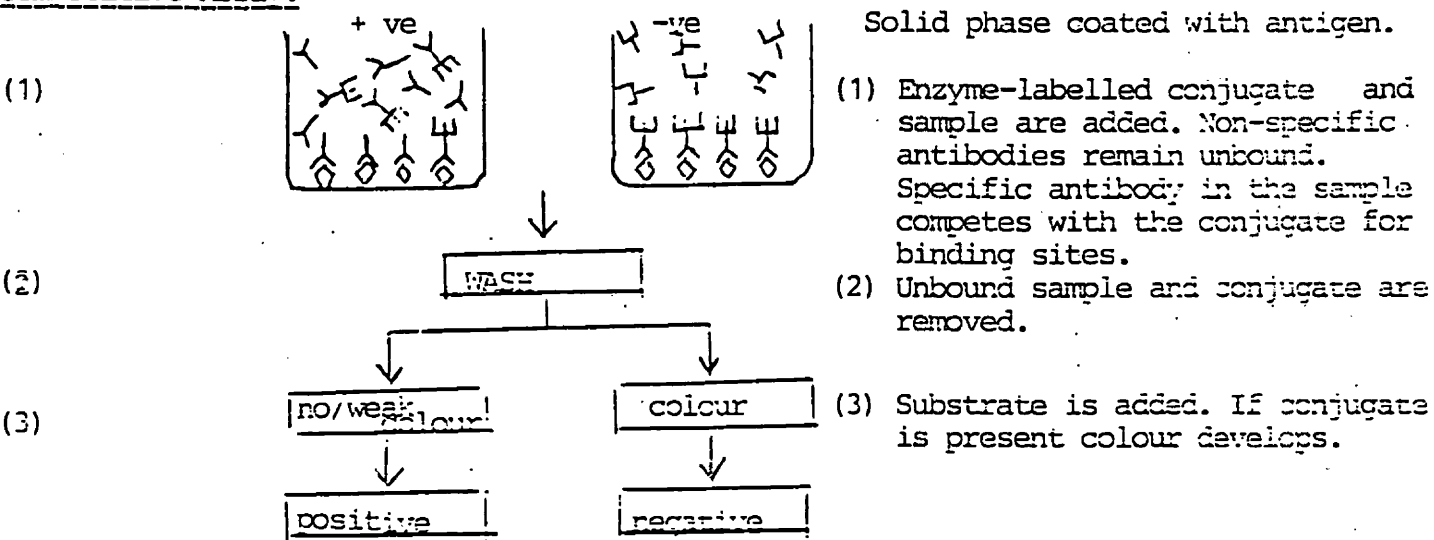
The principle of the "sandwich" type assay is described under enzyme immunoassays, (see Figure 11 (E)), and is very similar to that of SPRIA, except for the use of a radionuclide rather than an enzyme label. Antibody is bound to the solid phase (usually a microtitre well). Serum is then added and incubated in the well. Excess serum is washed away, leaving any antigen bound to the antibody on the solid phase. A labelled antibody is then added to form the antibody-antigen-antibody "sandwich" if antigen is present. When a radioisotope is used as the label, the quantity of bound antigen can be measured in a gamma counter.



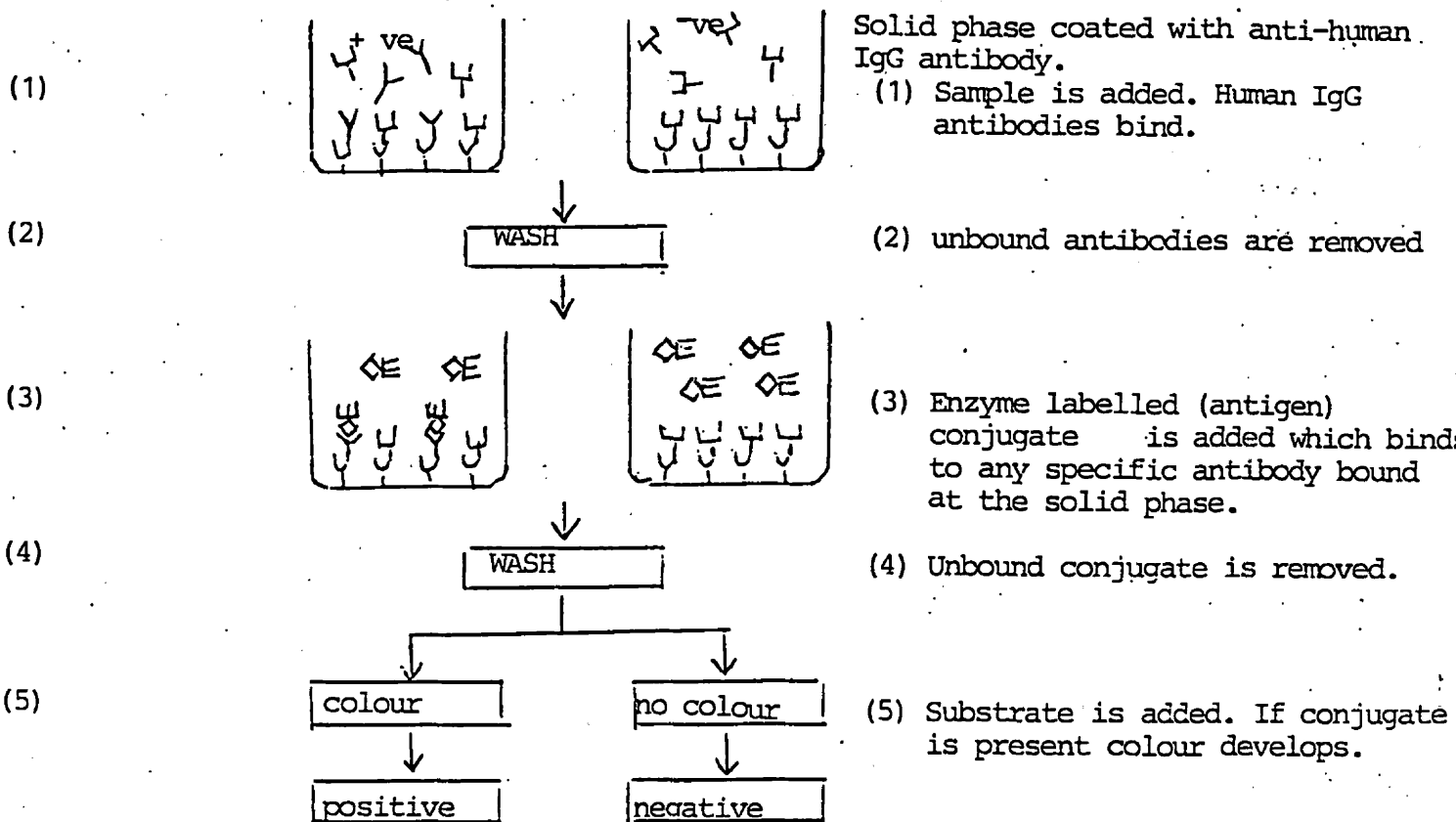
Indirect or Anti-globulin Assay.



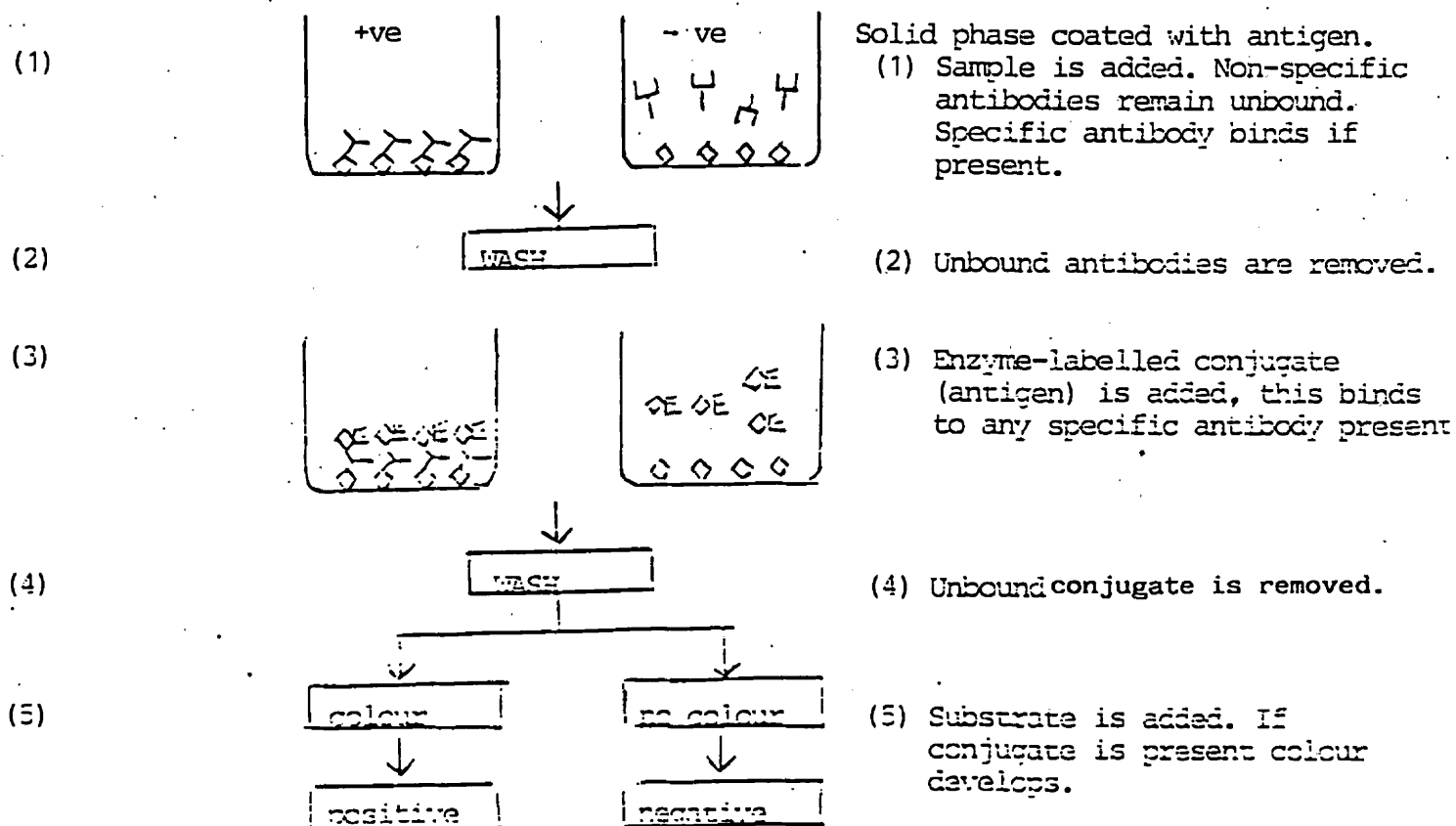
Competitive Assay.



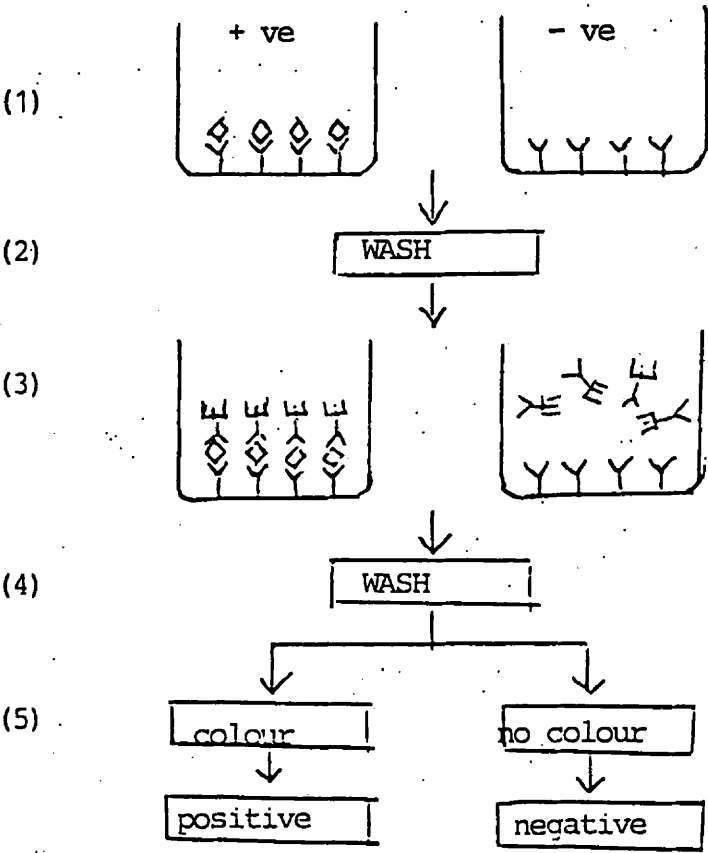
## Class-specific Antibody Capture Assay.



## Antigen Sandwich or Immunometric Assay.



Labelled Antibody Sandwich Assay.



Solid phase coated with specific antibody

- (1) Sample is added. Any antigen present is bound.
- (2) Unbound proteins are removed.
- (3) Enzyme-labelled conjugate (antibody) is added binding to any antigen bound at the solid phase.
- (4) Unbound conjugate is removed.
- (5) Substrate is added. If conjugate is present colour develops.

### Enzyme immunoassays.

Recent technological developments in HBV testing include the widespread use of monoclonal antibodies particularly in the enzyme-linked immunosorbent assay (ELISA), which avoids the use of radioactive labels and has a longer shelf-life. At NLBTC we currently use an EIA (enzyme immunoassay) marketed by Launch Diagnostics which employs polyclonal antibodies to HBsAg raised in guinea-pigs and goats. Assay time can be shortened by the use of a microplate shaker-incubator to only 55 minutes. (For full protocol see appendix 3.)

Using enzymes as a label in immunoassays avoids the use of controversial radioisotopes. A range of enzymes and associated substrates are available e.g. horseradish peroxidase with orthophenylene diamine (OPD) or tetramethylbenzidine hydrochloride (TMB) as substrate, or alkaline phosphatase with p-nitrophenyl phosphate as substrate. The resulting colour produced by enzyme and substrate as a chromogen can be read photometrically. For diagrams of the various types of enzyme immunoassays in current use see figure 11(A-E).

"Amplified" EIA systems are available, for example the Wellcozyme HBsAg EIA assay. In this system, alkaline phosphatase bound to the labelled antibody converts an NADP substrate to NAD. When the amplifying agent (alcohol dehydrogenase plus diaphorase) is added, NAD is converted

to NADH in the reaction between alcohol dehydrogenase and ethanol. This NADH reacts with iodonitrotetrazolium violet to form a coloured complex. NAD formed after the reaction can be converted back to NADH; because substrate and enzyme are in excess the reaction can cycle and amplify the signal.

### Chemiluminescence.

The use of a chemiluminescent label in immunoassays, as an alternative to radioisotopes, has attracted considerable interest in recent years. Amersham International have developed a range of chemiluminescence assays, using antibody labelled with horse-radish peroxidase which reacts with luminol as a signal-generating reagent. The system can be enhanced up to 100-fold by the use of an enhancer such as firefly luciferin, p-iodophenol or p-phenylphenol. This new labelling system is rapid, light emission is stable and sensitivity is improved. (Kricka and Thorpe 1985).

Unfortunately the company may not have aimed primarily at the transfusion service market with these assays: when evaluations were performed at NLBTC, the level of sensitivity of the HBsAg assay was less than that soon to be required by the Blood transfusion Service, i.e. 0.5 ng/ml. In our own lab we aim to detect a minimum of 0.25 ng/ml of HBsAg, and many current HBsAg assays will detect as little as 0.1 ng HBsAg/ml.

## Constraints of immunoassay systems.

### (a). Radioimmunoassay.

Isotopes with a short half-life such as  $^{125}\text{I}$  have a limited shelf-life of only a few weeks, this is the major disadvantage of this assay system, as a fresh preparation is required in order to achieve consistently high enough counts for detection purposes. This fact, and also the need to comply with the rigorous requirements of radiation protection regulations, are the main reasons why RIA is losing popularity and EIA is becoming increasingly more popular in the diagnostic world.

Radiolabels exhibit very low background noise levels leading to full realisation of sensitivity. Radioactive disintegrations remain unaffected by factors such as pH, concentration or temperature, leading to a more robust type of assay than EIA with a greater degree of specificity.

I feel personally that the decline in popularity of the RIA for HBsAg is to be regretted because there is still much advantage to be gained in the use of this simple, clean and sensitive technique. Product liability constraints forced us to abandon this technique due to lack of adequate quality control of the solid-phase coating procedure, whilst the assay was still of great diagnostic value.

In immunoradiometric assays the biphasic response or "hook" effect can be demonstrated at high antigen concentrations. (Also referred to as the "prozone" effect.) This introduces a problem into the assay of interpretation of results from high concentrations of antigen (as there is an ascending and descending curve produced) resulting in counts from the solid-phase bound antibody correlating with two different concentrations of antigen. At very high concentrations of antigen the descending curve can reach a point corresponding to the values for very low concentrations.

The sequential addition of reagents together with the removal of unreacted species between each reagent addition by washing will eliminate the "hook" and replace it with a plateau thus removing any ambiguity in the result.

#### Enzymeimmunoassay.

One of the problems of EIA is non-specific protein binding. This can be reduced by the addition of BSA to buffers. Some high backgrounds can be due to cross-reactivity or to low levels of natural antibodies present in reagents used. BSA or other serum proteins can be used to block non-specific binding to the solid phase but test samples may contain antibodies to the blocking protein itself. I actually observed this phenomenon personally in the use of the Biokit HBsAg assay and further purification of the protein was required to eliminate it.

Reaction rates are dependent upon temperature and various restraints must be placed upon incubation procedures to ensure minimisation of variation, such measures include not stacking plates, the use of heating blocks and the selection of suitable lengths of incubation to ensure well contents attain the correct temperature, thus avoiding the "edge" effect where wells at the edge of the plate demonstrate higher readings than those at the centre of the plate due to failure of the central wells to attain sufficiently high temperatures.

Occasionally the presence of enzyme inhibitors or natural enzymes such as peroxidases in the serum may affect the readings.

One serious constraint of the EIA system is that it is not really technically possible to see an absorbance reading greater than 2.0 (although some artifacts may be observed practically) since this would constitute 100% absorbance. This causes problems in quantitation, as the range of measurement is relatively small when compared with that of RIA and chemiluminescence assays which have a substantially higher upper range of measurement.

The vast majority of commercial HBsAg assays are now EIA systems, with the constraints placed upon us by the Medicines Inspectorate, the Blood Transfusion Service has less choice as to the type of assay used these days.



## Chemiluminescence.

I feel it is a great pity that the manufacturers of this type of HBsAg assay have not put enough effort into providing the type of assay required by the Blood Transfusion Service. There is great potential for quantitation using this assay, and the shorter incubation times required would be most useful for the quick release of blood.

One of the earlier constraints of this assay was the short duration of the signal, making it unsuitable for large batches of samples. This has now been overcome by the use of an enhancer such as firefly luciferin which can increase the signal by up to 600 times and also helps to depress the signal/noise ratio.

This assay is currently more prone to the problems of naturally occurring peroxidases in the serum than other enzyme catalysed assays.

Chemiluminescence assays are not subject to the high dose "hook" effect until very high (up to 600 ug/ml) levels of HBsAg are reached.

Table to summarise the constraints of RIA, EIA and Chemiluminescence.

<u>RIA</u>	<u>EIA</u>	<u>Chemiluminescence</u>
1.Short half-life of reagents.	1. Non-specific protein binding.	1.Need to enhance signal.
2.Radiation hazard regulations.	2. Cross-reactivity and natural antibodies.	2.Prone to problems with naturally occurring peroxidases.
3."Hook" effect at high antigen concentrations.	3. Antibodies to blocking protein	
	4. Temperature variations.	
	5. Enzyme inhibitor may be present.	

## Polymerase chain reaction.

Although it is not yet applicable to routine donor screening, great success in a research context has been achieved using the polymerase chain reaction (PCR) (Schochetman et al. 1988) which can allow geometric amplification of a target sequence of DNA (Figure 12.). The resulting "amplified" DNA can then be detected by conventional methods.

In PCR, sequences of DNA or RNA are amplified in vitro over a million times in just a few hours. If an RNA sequence is to be amplified, a DNA copy of it must be synthesised by using reverse transcriptase before the PCR is begun. The principle of PCR is simple. Two oligonucleotide primers that flank the DNA segment for amplification are used to direct DNA synthesis in opposite and overlapping directions. The procedure involves repeated cycles of heat denaturation of the DNA, annealing of the primers to their complimentary sequences and extension of the annealed primers by DNA polymerase. The primers are oriented so that synthesis of the DNA by the polymerase proceeds across the region between them. Each cycle of heating, cooling and polymerisation doubles the number of copies of the target DNA.

In early procedures, an enzyme known as the Klenow fragment, derived from Escherichia coli, was used to build the new chains of DNA. This caused problems because the high temperature required to separate the two strands of a

double helix also destroys the ability of the enzyme to catalyse polymerisation. This necessitated the addition of fresh enzyme at each stage in the reaction. Recently a better solution has been described (Science, vol 239, p487) in which a polymerase derived from the bacterium Thermus aquaticus (Taq), which lives in hot springs and geysers, is used. This polymerase survives extended incubation at 95°C. One addition of enzyme is able to keep working throughout all cycles of DNA amplification. Running the PCR at a higher temperature has made the reaction more sensitive and more specific resulting in less amplification of non-target sequences, as well as giving a higher yield.

The advantages of the polymerase chain reaction lie in its high degree of specificity, sensitivity and also its simplicity and directness in comparison with previous methods of gene isolation and purification. This method potentially lends itself to diagnosis of infectious diseases because it does not require radioactive detection systems. In fact, if sufficient genomic material is available, diagnosis can be made by direct visualisation of the amplified sequences by staining with ethidium bromide after gel electrophoresis. The higher degree of specificity afforded by this technique permits the detection of viral material even when there is a large excess of host nucleic acid. However at this stage the technique is still too expensive for routine use.

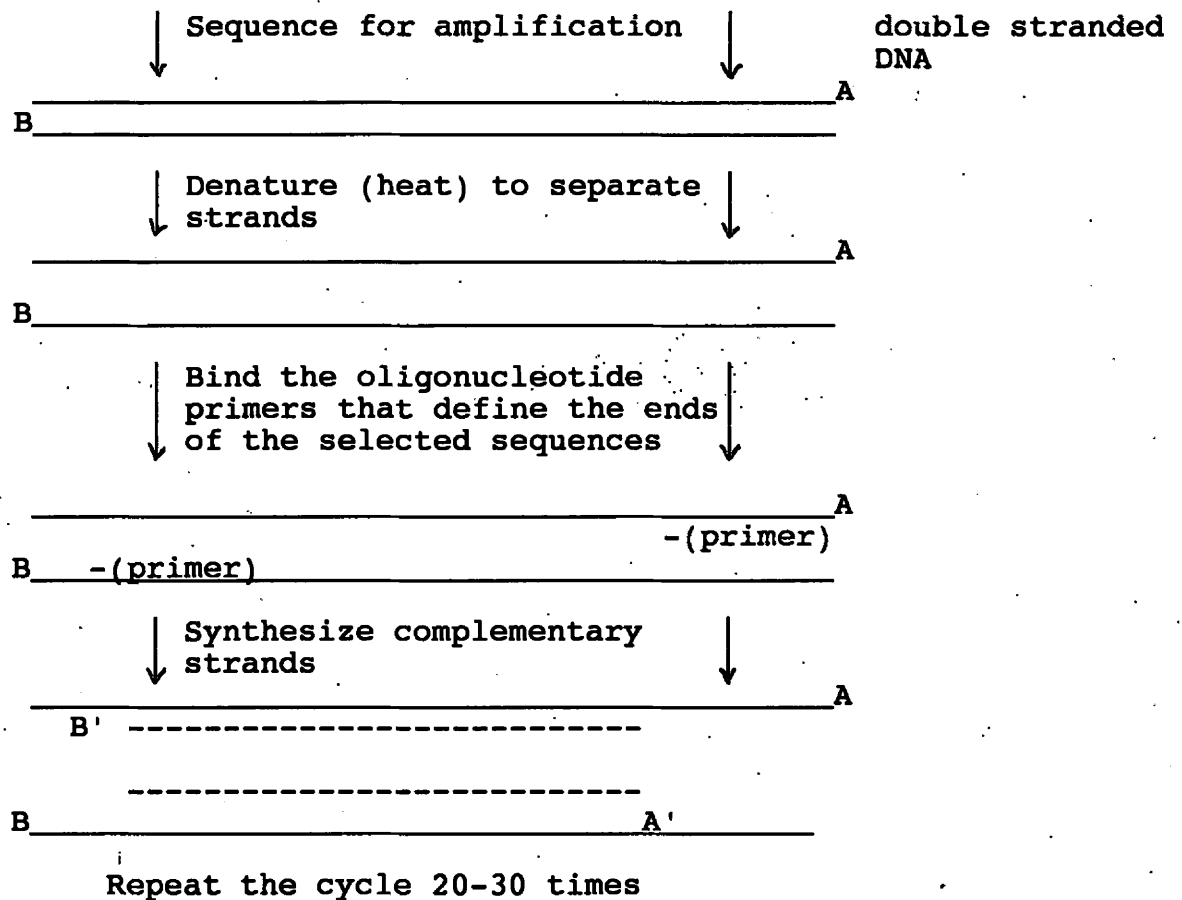
PCR amplification lends itself well to automation and promises a rapid detection method for an enormous number

and variety of clinical applications although not yet as a screening test in the transfusion context. A PCR assay for the detection of HBV has been developed and used by research workers in apparently healthy seropositive and seronegative Chinese individuals ( Pao et al. 1991) who have a HBsAg prevalence rate of 25%. But the feelings are that at the present time the PCR assay probably belongs in the research laboratory until standardised assays are available and specificity problems are resolved. This type of assay could in fact be too sensitive in a transfusion context, as it would also amplify non-replicating genomic material in immune individuals. A more suitable method for DNA detection in this context would be the less sensitive DNA hybridisation technique. (Dot blotting.).

#### DNA Hybridisation.

Viral DNA in a specimen is spotted on to a nitrocellulose membrane where it is bound and denatured with alkali. It is then hybridised with a radioactively labelled viral DNA probe and autoradiographed.

Figure 12. Mechanism of polymerase chain reaction.



In order to prevent the transmission of HBV to a vulnerable population it is necessary to have confidence in the sensitivity, specificity and reproducibility of the tests used to screen donor blood for the various markers of viral infection prior to transfusion. The following section is an evaluation of the assays and an attempt to assess the degree of confidence we can place upon them.

**SECTION 3: EXPERIMENTAL EVALUATIONS OF THE ASSAYS EMPLOYED**

**IN**

**TRANSFUSION MICROBIOLOGY.**



### 3:1 A comparative evaluation of commercially available

#### HBsAg EIA assays.

During the course of this study the routine method of HBsAg screening at NLBTC had to be changed. The BPL RIA assay was no longer available to us because of the problems associated with the use of radioisotopes and also because in order to achieve the required level of sensitivity the assay needed to be run overnight. This was considered too long to allow for the rapid release of blood for transfusion.

It fell to me personally to examine some of the commercially available HBsAg diagnostic kits on the market with a view to their suitability in the context of blood transfusion.

When assessing a diagnostic kit it is necessary to take several factors into consideration. It is important to achieve a balance between sensitivity, specificity, cost and convenience. I firstly canvassed other Blood Transfusion Centres to see which assays they were using and what sort of results they were obtaining in order to narrow down the number of assays evaluated. I then set about running a trial batch of 5 coated microplates for each assay under trial. Each plate contained both donor samples and a range of known standards, specifically the BPL standards calibrated to contain 0.1, 0.25, 0.5, 1, 4, 10, and 20 nanograms of HBsAg per ml.

Ideally, several forms of standard would have been evaluated at various dilutions and examined for parallelism to establish comparable performance with all assays.

However at the time I was restricted to the use of the BPL standards, therefore the comparison is perhaps not strictly as complete as it might have been, had more standards been available to me.

If the assay matched our requirements for sensitivity, i.e. to detect 0.2 ng/ml or less, using the BPL reference preparations, I then ran a further trial of 1000 donor sera to assess specificity. I include the results of these trials to give some idea of the range of HBsAg assays that are currently available.

#### Kits Evaluated.

1. Wellcozyme HBsAg amplified ELISA.
2. Biokit Bioelisa HBsAg. (Launch).
3. Fujirebio FRELISA HBs.
4. Amerlite HBsAg chemiluminescence assay  
(Amersham International).
5. Abbott Auszyme Monoclonal.
6. Behring Enzygnost HBsAg Monoclonal.
7. Mercia Heprofile HBsAg.

## 1. Wellcozyme HBsAg amplified ELISA.

### Kit Specifications.

The test is a type IV (see note on types of immunoassay) "sandwich" amplified ELISA which uses plates coated with a mouse monoclonal antibody to HBsAg. The conjugate is alkaline phosphatase-labelled mouse monoclonal antibody to HBsAg in a serum base. The substrate is NADP, and the amplifier contains alcohol dehydrogenase and diaphorase.

### Procedure.

The manufacturers offer alternative incubation procedures but the 37°C protocol was used for this evaluation as this corresponds to current laboratory practice in testing for other infectious agents, and is suitable for testing plasma as well as serum. (Higher incubation temperatures may not be used for plasma samples.) The incubation was carried out in an air incubator on a heating block inside a wet box.

There are three incubation periods and one washing stage. Total assay time is approximately two and a half hours including sampling, washing and reading.

Controls per plate are 4 negatives and 2 positives.

Reading is performed at 492 nm.

Calculation of the cut-off is performed by taking the mean of the negative controls and adding 0.1.

Observations.

The coefficient of correlation obtained for a standard curve employing the BPL positive standards was 0.9866.

The assay was sensitive to 0.2 ng/ml but the cut-off was set rather high and there was a noticeable difference between the negatives and the 0.1 ng/ml standards.

Extremely low levels of both AD and AY subtypes could be detected ( i.e. at a dilution of 1 in 300,000 ).

## 2. Biokit Bioelisa HBsAg. (Marketed by Launch Diagnostics).

### Kit specifications.

The assay is a type IV "sandwich" polyclonal ELISA using plates coated with guinea-pig anti-HBs, and goat anti-HBs conjugated with peroxidase. The substrate is hydrogen peroxide and the chromogen is TMB (tetramethylbenzidine).

### Procedure.

Incubation can be either overnight at room temperature or at 40°C in either a waterbath or an air incubator.

There are three incubation periods and two washes. Total assay time is just over two and a half hours including sampling, washing and reading.

Controls per plate are 2 blanks, 5 negatives and 1 positive.

Reading is performed at dual wavelengths of 450 nm with a reference filter of 620 nm.

Calculation of the cut-off is performed by taking the mean of the negatives and adding 0.04.

### Observations.

The coefficient of correlation obtained for a standard curve employing the BPL positive standards was 0.9937.

Difficulties were encountered when attempting to calibrate the plate reader against wells as directed by the manufacturer. Valid results were obtained if the reader was calibrated against air. The manufacturer confirmed that this was a valid procedure.

The lowest level of HBsAg to be 100% detectable for this assay was 0.25 ng/ml.

### Figure 13.

Summary table of eight plates: trial run Biokit HBsAg EIA.

Plate number	mean cut-off	number of reactivities	"grey zone" reactivities	percentage reactive rate
1	0.159	2	0	2.3
2	0.154	0	0	0.0
3	0.160	1	1	1.2
4	0.134	0	5	0.0
5	0.128	0	1	1.2
6	0.141	0	1	0.0
7	0.136	1	2	1.2
8	0.113	1	4	1.2
Total	0.141	5	14	0.7

Batch number J-4890

"grey zone" = cut-off +/- 10%.

A further evaluation of the test using six plates of donor sera resulted in two false positive reactions involving contamination of the well at substrate addition stage.

Trials of a short incubation version of this assay were done using the normal freeze-dried conjugate and the new liquid conjugate. Initially, sensitivity was reduced but on shaking the plates in a shaker-incubator, sensitivity was restored to a detection limit of 0.25 ng/ml. using the freeze-dried conjugate. The use of the new liquid conjugate improved the specificity of the test leading to a reduction in false-positivity.

### 3. FRELISA HBs (Fujirebio).

#### Kit Specifications.

The assay is a one-step "sandwich" method ELISA, using plates coated with a mouse monoclonal anti-HBs. The conjugate contains another mouse monoclonal anti-HBs and horseradish peroxidase. The chromogen is OPD. Therefore incubation with the substrate has to be performed in the dark.

#### Procedure.

The sample and conjugate are initially incubated for one and a half hours in an air incubator at 37°C. The substrate is added and the second incubation is for 30 mins. at room temperature.

Total assay time including sampling and reading is two and a half hours.

Controls per plate are 3 negatives and 2 positives.

Calculation of the cut-off is performed by taking the mean of the negative controls and adding 0.5.

Reading is by dual wavelength at 414/492 nm.

#### Observations.



The coefficient of correlation for a standard curve employing the BPL positive standards was 0.9905.

Standards less than 0.5 ng/ml were negative by this assay.

#### 4. Amerlite HBsAg. Amersham International.

##### Kit Specifications.

The assay is a type IV labelled antibody sandwich assay using a chemiluminescent enzyme as a label. Microplates coated with mouse monoclonal anti-HBs comprise the solid phase. The conjugate is a mouse monoclonal anti-HBs of a different specificity conjugated with horseradish peroxidase. The substrate contains luminol and a peracid salt.

##### Procedure.

The signal reagent, signal reagent buffer and the wash buffer are common to all Amersham assays and are not provided with the kit.

The wells are packed in strips and the plate has to be manually assembled before use. Assembly of a sufficiently flat plate surface suitable to pass easily through the washer and analyser is difficult to achieve as some of the wells are prone to protrude from the surface.

Initial incubation is one hour in the Amersham shaking incubator at 37°C. The plate is washed and the substrate containing the signal reagent is added and the plate can be read 2-20 mins after addition.

The plate is read in the Amerlite Analyser which provides a printout of all results in a choice of formats.

Total assay time including sampling, washing and reading is approximately one and a half hours.

Controls per plate are 3 negatives and 3 positives. However they are not in the conventional positions and the software is geared to these non-standard control positions at present.

Calculation of cut-off is performed by the analyser which normalises the signal. All readings greater than 1 are flagged as reactive.

#### Observations.

Sensitivity of this assay differs according to the subtype but 0.5 ng/ml must be considered as the lower limit of detection. Using the manufacturer's cut-off criteria.

This assay is fast but not as sensitive as others tested.

Larger scale clinical trials of this assay had been previously performed at NLBTC and extensive evaluation results are available.

## 5. Abbott Auszyme Monoclonal.

### Kit specifications.

The assay is an EIA using a coated bead as the solid phase. The bead is coated with a mouse monoclonal anti-HBs. The conjugate is mouse monoclonal anti-HBs peroxidase (HRPO). The substrate is OPD.

The assay uses the Abbott Commander flexible pipetting centre and parallel processing centre. The beads are incubated in the Abbott plastic trays and the system is not compatible with the microtitre system. Abbott provide an air incubator and a "dynamic" or shaking incubator is shortly becoming available.

### Observations.

The initial incubation of sample and conjugate is 75 mins. at 40°C .(There are three different protocols including an overnight and a three hour incubation). Incubation with the substrate takes 30 mins at room temperature under cover to exclude light.

Dispensing of reagents is performed by the processor and all reading is done inside the processor.

The total assay time excluding pipetting was just over two hours. However the use of an automated sampler would increase the processing time accordingly, but would add the

benefits of barcoded trays and positive sample identification.

Controls per run are two positives and three negatives but this one set of controls can be used for up to ten trays in a batch.

Reading is performed in the processor at 492 nm within two hours of the addition of 1N sulphuric acid, again performed by the processor.

Calculation of the cut-off is performed by taking the mean of the negatives and adding 0.025.

#### Observations.

The correlation coefficient obtained for the BPL standards was 0.9976.

The assay was able to detect the 0.1ng/ml standard in 11 out of 12 replicates.

A 1000 test trial-run performed in the lab resulted in only 1 false positive result.

## 6. Behring Enzygnost Monoclonal.

### Kit specifications.

The test is a one-step EIA using plates coated with sheep anti-HBsAg. The conjugate is monoclonal anti-HBs conjugated with peroxidase and preserved with phenol max.1g/l. The substrate is TMB.

### Procedure.

Initial incubation is for 90 minutes at 37°C in either air or water but water is preferred. There is only one washing stage and the second incubation is in the dark at room temperature.

Total assay time including sampling and washing is approximately two and a half hours.

Controls per plate are 4 negatives and 2 positives.

Reading is at 450 nm.

Calculation of the cut-off is performed by taking the mean of the negative controls and adding 0.05.

Observations.

The coefficient of correlation for a standard curve using the BPL positive standards was 0.9839.

The limit of detection for this assay was 0.25 ng/ml.

Behring have available a fully automated processing system, and full hardware and software back-up is also available.

## 7. Mercia Heprofile HBsAg.

### Kit specifications.

The test is a type IV "sandwich" ELISA which uses plates coated with mouse anti-HBs. The conjugate contains peroxidase and chimpanzee anti-HBs. The substrate is hydrogen peroxide and the chromogen is TMB.

### Observations.

Incubation times vary with accompanying variations in sensitivity. There is a 90 min. incubation, a two and a half hour incubation and an overnight incubation. The 90 min incubation is sensitive only to 0.5 ng/ml. The two and a half hour incubation is claimed to be sensitive to only 0.25 ng/ml, but in the trial run it was possible to detect four out of eight replicates of 0.1 ng/ml. The overnight incubation is said to detect 0.125 ng/ml but was not attempted in this trial. Incubation may be carried out in air or in a waterbath at 37°C or at room temperature overnight.

There are three incubation periods and two wash steps. The shortest possible assay time would be one and a half hours including sampling and washing.

Controls per plate are 3 negatives and 2 positives.



Reading is at 450 nm.

Calculation of the cut-off is performed by taking the mean of the negative controls and adding 0.05.

#### Observations.

The coefficient of correlation obtained for the BPL positive standards was 0.9886.

In our hands, the sensitivity was better than that predicted by the manufacturers, detecting four out of eight replicates of the 0.1 ng.ml standard when using the two and a half hour protocol.

Figure 14.

Tables of Reproducibility.

1.

Wellcozyme HBsAg.

ng/ml	0.1	0.25	0.5	1	4	10
n	11	11	8	10	18	8
mean OD	0.235	0.309	0.394	0.562	1.489	2.765
SD	0.013	0.019	0.137	0.043	0.079	0.082
CV	5.53	6.15	5.398	7.65	5.3	2.99

Cut-off = 0.283

2.

Biokit Bioelisa HBsAg (Launch).

ng/ml	0.1	0.25	0.5	1	4	10
n	16	16	8	8	8	10
mean OD	0.072	0.1196	0.179	0.313	1.015	1.997
SD	0.0039	0.005	0.0085	0.012	0.028	0.036
CV	5.44	4.28	4.73	3.977	2.78	1.79

Cut-off = 0.0928

Tables of reproducibility cont.

3. Abbott Auszyme.

ng/ml	0.1	0.25	1	4
n	12	12	10	12
mean OD	0.04	0.09	0.28	0.86
SD	3.57	6.48	0.013	0.06
CV	8.94	6.95	4.55	7.01

Cut-off = 0.032

4. Behring Enzygnost.

ng/ml	0.1	0.25	0.5	1	4
n	6	6	6	6	6
mean OD	0.117	0.16	0.232	0.36	1.01
SD	0.012	0.014	0.117	0.02	0.05
CV	10.08	8.48	5.07	6.16	5.17

Cut-off = 0.148

Tables of Reproducibility cont.

5. Mercia Heprofile.

ng/ml	0.1	0.25	0.5	1	4
n	8	8	8	8	8
mean OD	0.202	0.28	0.46	0.82	2.497
SD	0.011	0.017	0.036	0.03	0.08
CV	5.388	5.97	8.02	3.86	3.2

Cut-off = 0.201

## Summary of methods of calculation of cut-off in HBsAg Assays.

I include this brief summary to illustrate that whatever careful trials and evaluations are performed in the laboratory, the ultimate decision of a positive or negative result is set by the manufacturer by the use of a "factor" based on calculations performed in their own Q.C. department! It would be possible also to set the "cut-off" from the mean of the negative samples on the plate plus two standard deviations, thus reflecting the true performance of the assay. Care must be taken to exclude outliers. Some sera, through non-specific binding (see page 86) give falsely high readings leading to an artificially high cut-off and hence reducing sensitivity. Equally, falsely low readings would lead to an artificially low cut-off and a resulting loss of specificity. This method has been considered as an option at NLBTC but not implemented because, due to product liability constraints, we are required to adhere strictly to the manufacturer's criteria for the establishment of the cut-off.

### 1. Wellcozyme.

Take the mean of the negative controls and add 0.1.

### 2. Biokit Bioelisa.

Take the mean of the negative controls and add 0.04.

### 3. Fujirebio FRELISA.

Take the mean of the negative controls and add 0.5.

### 4. Amerlite HBsAg.

Calculation of the cut-off is performed by the Amersham Analyser, which normalises the signal and flags all readings greater than 1.

5. Abbott Auszyme.

Take the mean of the negatives and add 0.025.

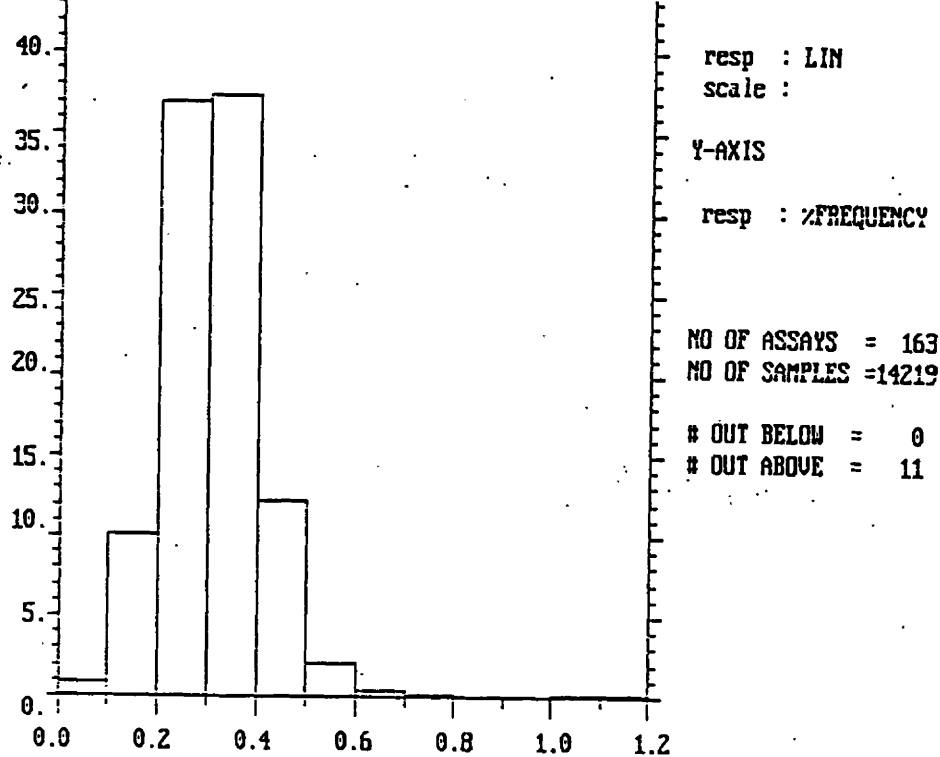
6. Behring Enzygnost.

Take the mean of the negative controls and add 0.05.

7. Mercia Heprofile HBsAg.

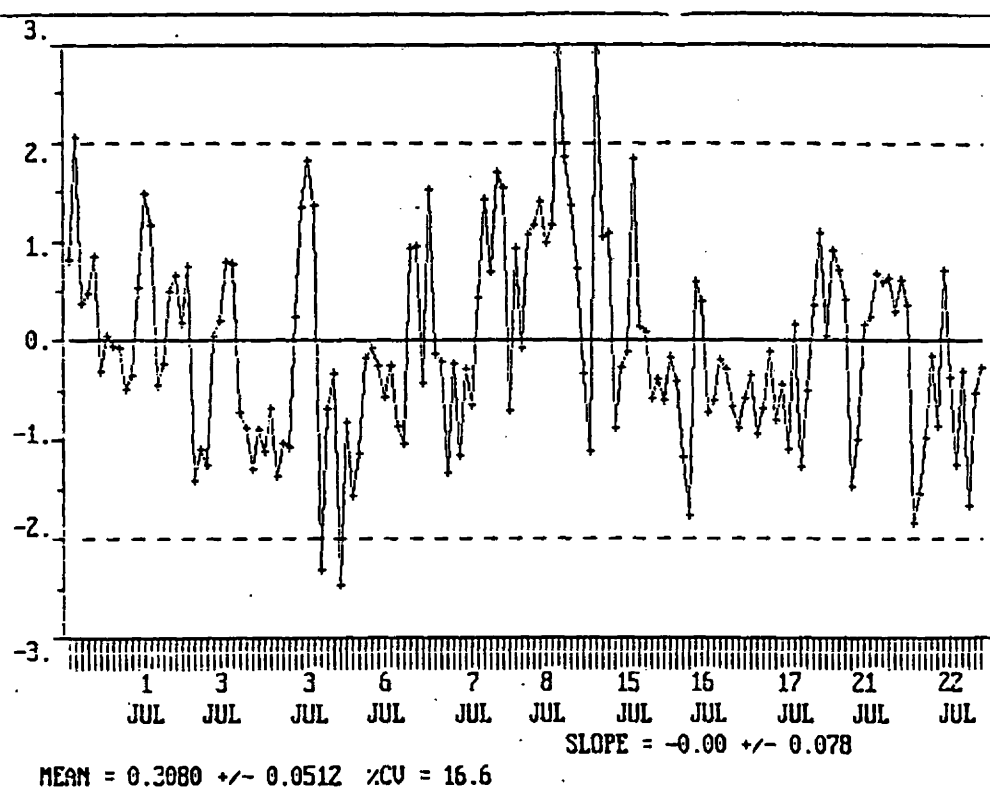
Take the mean of the negative controls and add 0.05.

I feel that we have chosen the most appropriate assay method for our requirements because, not only does the assay meet national requirements for sensitivity, but also, most of the reactives we see are genuine neutralisable positives. Our initial reactive rate currently stands at 0.08% which by any standards is a very clean assay, but particularly so with an EIA assay (see page 86). It is now very rare to see a repeatable false-positive reaction by this assay. However, this cannot be said of all EIA assays evaluated (see previous data ). Non-repeatable reactives are usually due to technical errors.



**Figure 14 (a).**

Typical distribution of negatives plus 11 positives on 163 assays performed using the Biokit Bioelisa HBsAg assay. The results are normalised to the cut-off hence anything greater than 1 is positive.



**Figure 14 (b).**

Distribution of the means of the negative samples on the same 163 assays most fall within two standard deviations of the mean, 5 are outside this range reflecting variation in incubation conditions as the plates are batched before incubation.

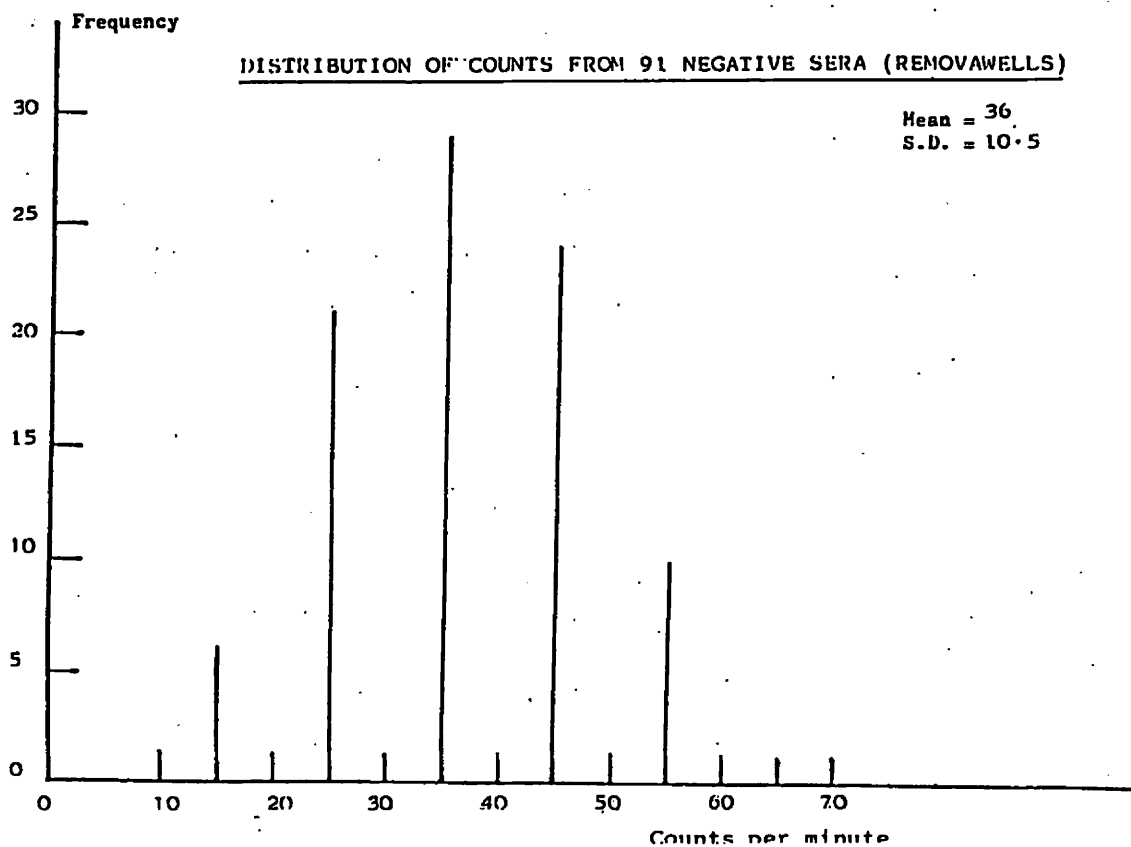


Figure 14 (c).

Typical distribution profile of negative samples using the SPRIA for HBsAg. My thanks to Dr. David Howell for this illustration.



### 3:2 Evaluation of assays used in this study.

In order to affirm the validity of the data collected, it was necessary to perform an evaluation of the reproducibility and precision of the main assays used in the provision of the data over the years. This was in order to show that where changes in titre or where seroconversions had occurred, we were looking at significant differences and not just normal or experimental variations.

#### Evaluation of precision and reproducibility of RPHA assay.

Aim: To perform a series of end-point titrations on a sample of known HBsAg titre (1:64 by 1% Hepatest) and to observe the reproducibility of the test under standard laboratory procedures.

Method: Two operators worked in parallel for this evaluation. The 1:64 standard was titrated 10 times in parallel by 1% Hepatest (Wellcome Diagnostics) by both operators on one day using a single batch of test reagents. The next day, both operators repeated the same procedure again using the same batch of reagents.

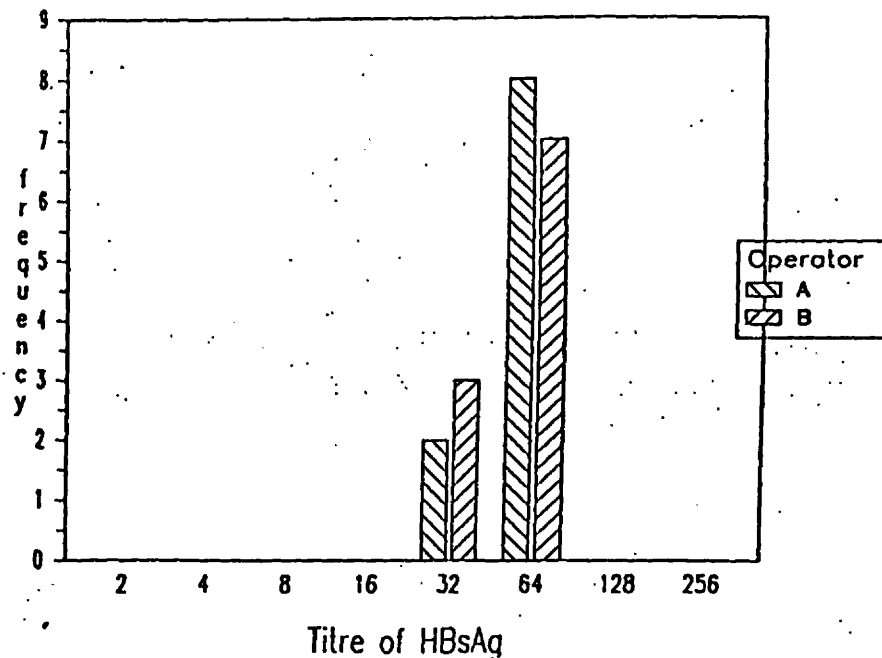
After an interval of one week, the procedure was again repeated, this time using a different batch of reagents.

Titration. 0.025 ml of kit buffer was added to the wells of two U-well microtitre plates per operator. The test and control cells were reconstituted in 0.025 ml of kit buffer as per manufacturer's instructions. 0.025 ml of the standard was serially diluted in the wells of the microtitre plates to give dilutions of 1:2, 1:4, 1:8 etc. until the final well gave a dilution of 1:256. This was repeated in two rows of wells for each sample tested to give a row of test dilutions and a row of control dilutions. This test was repeated using the same standard, five times in each plate. The kit positive control serum was then similarly diluted in the last two rows of each plate, and with a 0.025 ml dropper, 0.025 ml of the test and control cells were added to alternate rows of the plates. The plates were allowed to settle undisturbed on the bench for 30 minutes until the agglutination patterns were visible in the bottoms of the wells.

Interpretation of results.

A positive reaction was deemed to be one in which a smooth carpet of cells could be seen at the bottom. A negative well was one in which a tight button of cells could be seen at the bottom. Reference was made to the positive control titration to help determine the reaction pattern that would be taken as the " cut-off ".

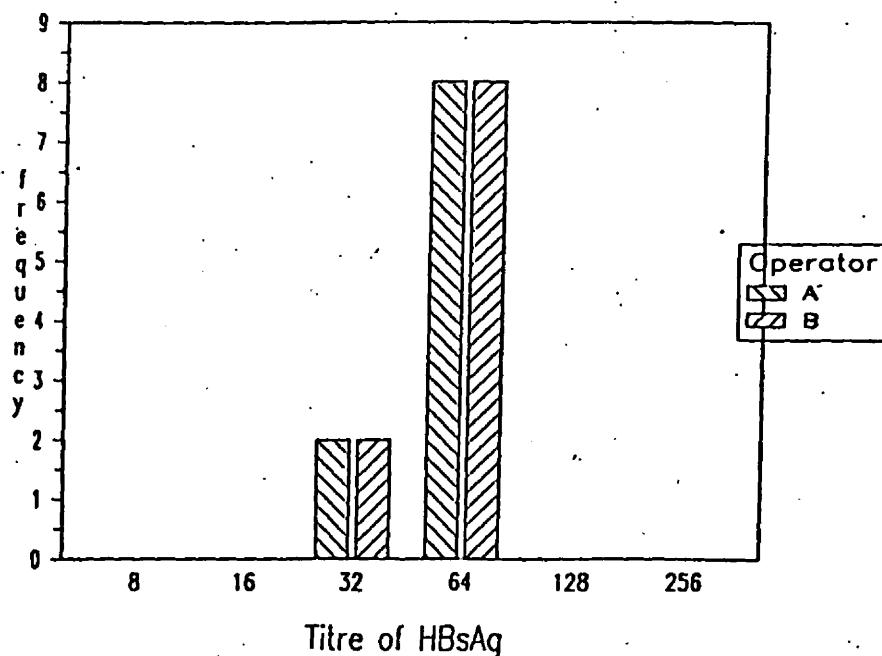
**Figure 15.**



Batch no. K158610 run 2

Reproducibility of HA assay

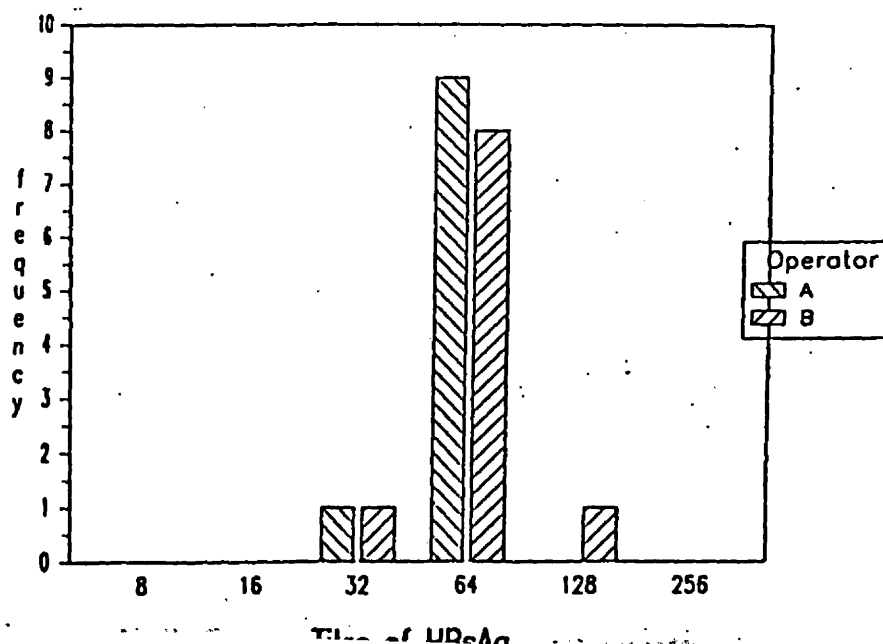
**Figure 16.**



Batch no. K634310

Reproducibility of HA assay

**Figure 17.**



Results: The results of these test runs were compared and analysed (see figures 15-18.)

Figure 18: Summary of Haemagglutination Evaluation Result

Run No.	Operator 1			Operator 2		
	Frequency	Titre	C.V.	Frequency	titre	C.V.
1	8	64		8	64	
	2	32	6.8	2	32	6.8
2	8	64		7	64	
	2	32	6.8	3	32	8.7
3	9	64		8	64	
	1	32		1	32	
			5.1	1	128	7.4

Batches used were K15861 for runs 1&2, and K634310 for run 3.

Comment. Coefficients of variation are as in figure 18. The differences in them are very small: i.e. between 5.1 and 8.7. but this is not directly comparable with those for the RIA assay since the HA is logarithmic.

## Evaluation of Precision and Reproducibility of SPRIA Assay.

Aim : To perform a series of replicate RIA tests on a sample of known HBsAg content (1ng/ml B.P.L. Elstree) and to observe the spread of counts per minute under standard laboratory procedures.

Method : A sufficient number of "U" wells were coated under normal laboratory conditions with horse anti-HBs 1/1000 (B.P.L. Elstree) for 3 days at room temperature, and then with bovine serum albumin for 2 hours at room temperature.

0.1 ml of the 1ng/ml standard was inoculated into each test well.

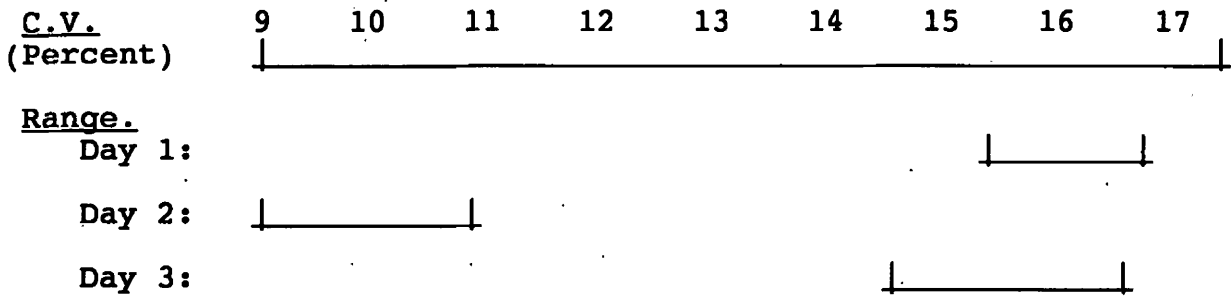
The plates were then incubated for 2 hours in a waterbath at 45°C. They were then washed x 5 in deionised water, and 0.1 ml of  $^{125}\text{I}$  goat anti-Hbs label was added to each test well. The plates were re-incubated in the waterbath for a further 2 hours before being rewashed x 5 in deionised water. The wells were then separated and counted in a 16-channel gamma counter for 60 seconds.

The above procedure was repeated on three separate runs by the same operator (the author), at intervals of one week, using two replicate plates of 96 wells, to give a good range of test values.

### Results:

Statistical analysis was performed on the data obtained.

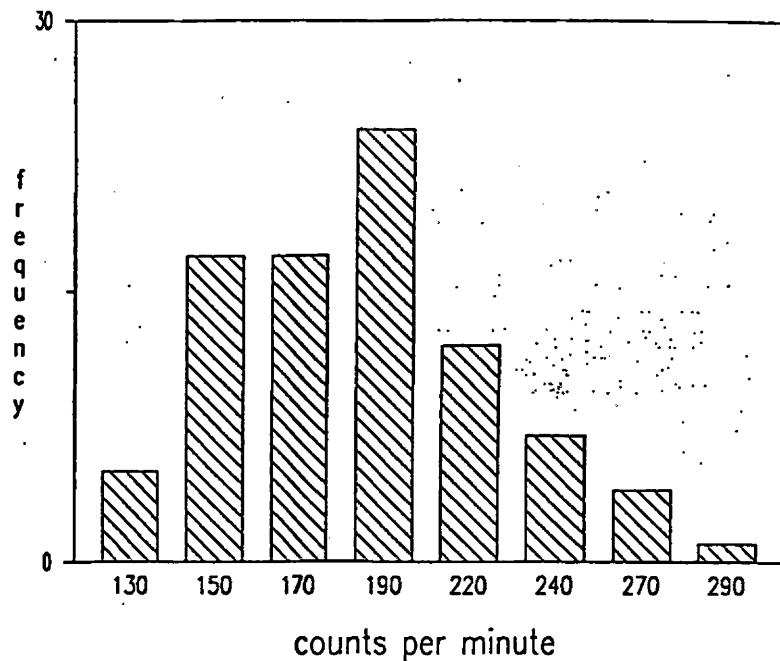
**Figure 19:** Diagrammatic representation of C.V. ranges on each day.



Comment:

Within a run, coefficients of variation vary by 2% or less (i.e. little variation). However, on different days the C.V.s can vary by as much as 8%. In the context of biological assay variation, however, 8% is a very favourable value considering that titrations such as RPHA are considered to have an accuracy of +/- 1 well (i.e. +/- 100% variation) 16--32--64. It was therefore concluded that a significant difference in RPHA titre would be a fall ( or rise) in titre by three wells or more. Variation of one, or even two wells could be construed as experimental variation. RIA, or equivalent immunoassay, would therefore be the assay of choice for the examination of significant falls in HBsAg quantitation as RPHA titres are less accurate.

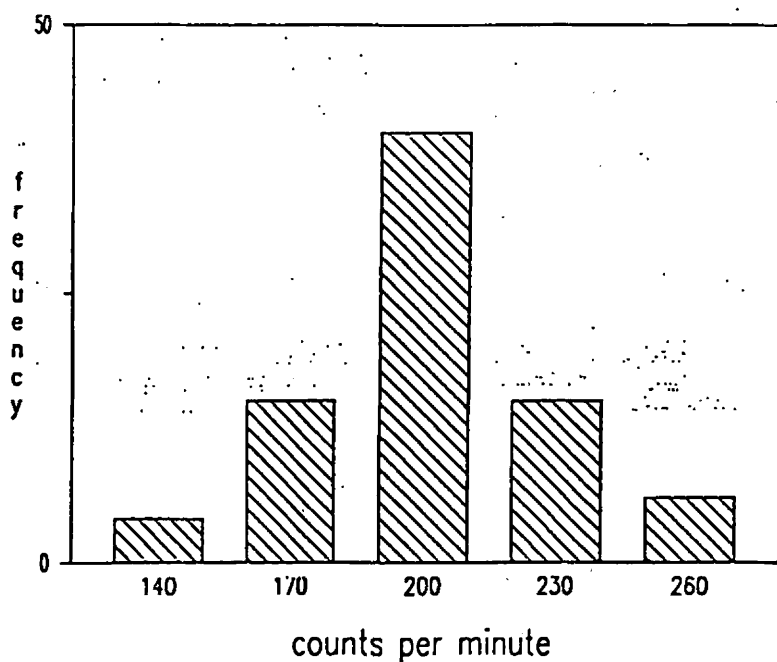
Figure 20.



Run 1 Plate 2

Distribution of counts per min

Figure 21.



Run 2 plate 1

Distribution of counts per min

Figure 22.

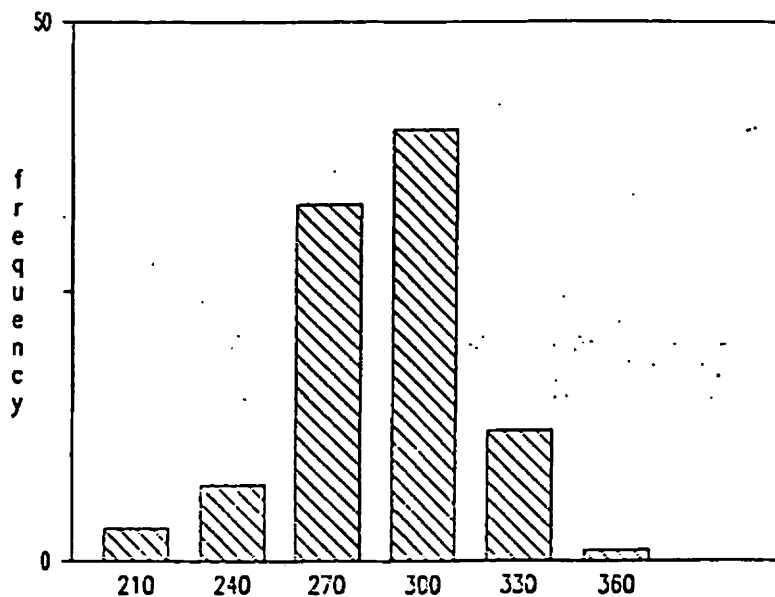
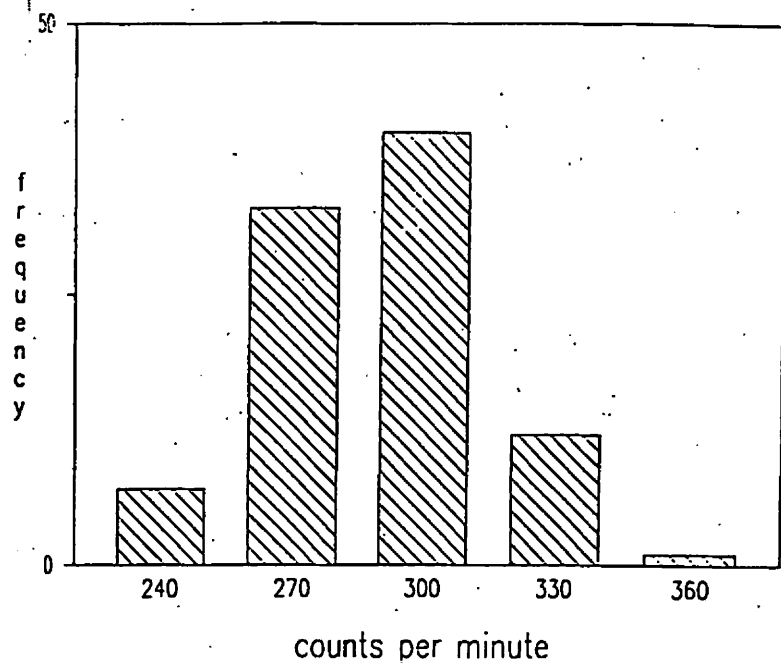


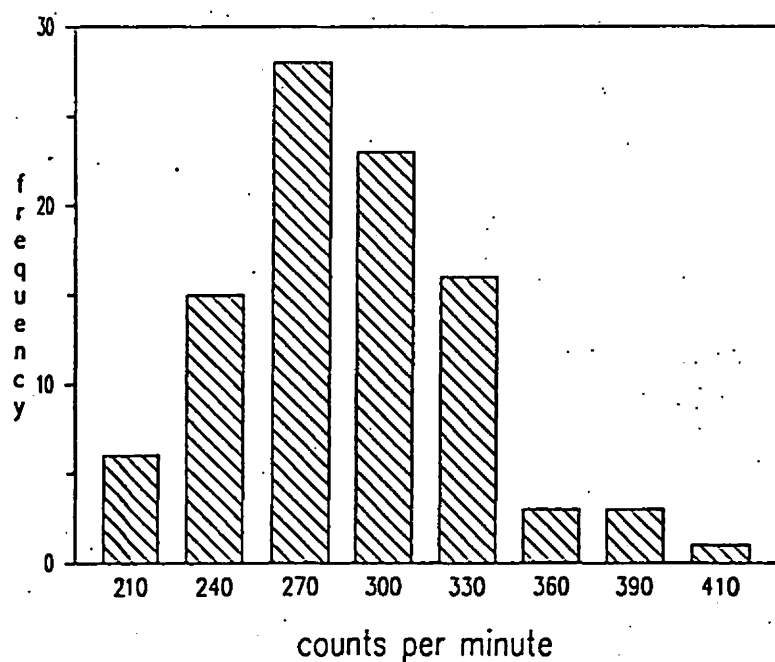
Figure 23.



Run 3 plate 1

Distribution of counts per min

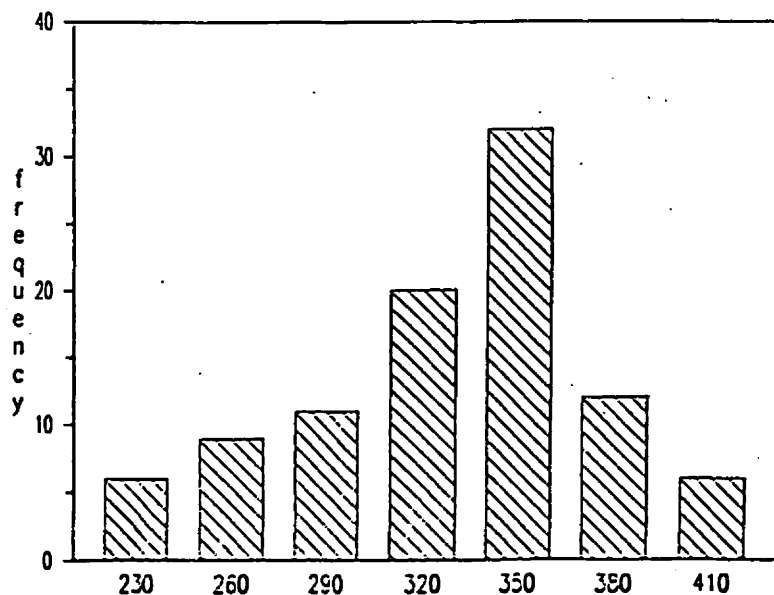
Figure 24.



Run 3 plate 2

Distribution of counts per min

Figure 25.





**Figure 26.**

**Statistical Evaluation of RIA test**

Day No	Plate No.	Mean cpm	S.D.	C.V.	95% conf. Limits of Values	Se	95% conf. limit of mean
1	1	204.66	30.81	15.05	143.04-266.28	3.14	201.52-207.8
	2	192.56	32.07	16.65	128.42-256.7	3.3	189.26-195.86
2	1	355.9	39.2	9.1	277.5-434.3	4.0	351.9-359.9
	2	290.8	28.2	10.32	234.4-347.2	2.9	287.7-293.7
3	1	290.6	42.2	14.5	206.2-375.0	4.3	286.3-294.9
	2	329.9	54.4	16.5	221.1-438.7	5.6	324.3-335.5

Q.C. Calibration Curve

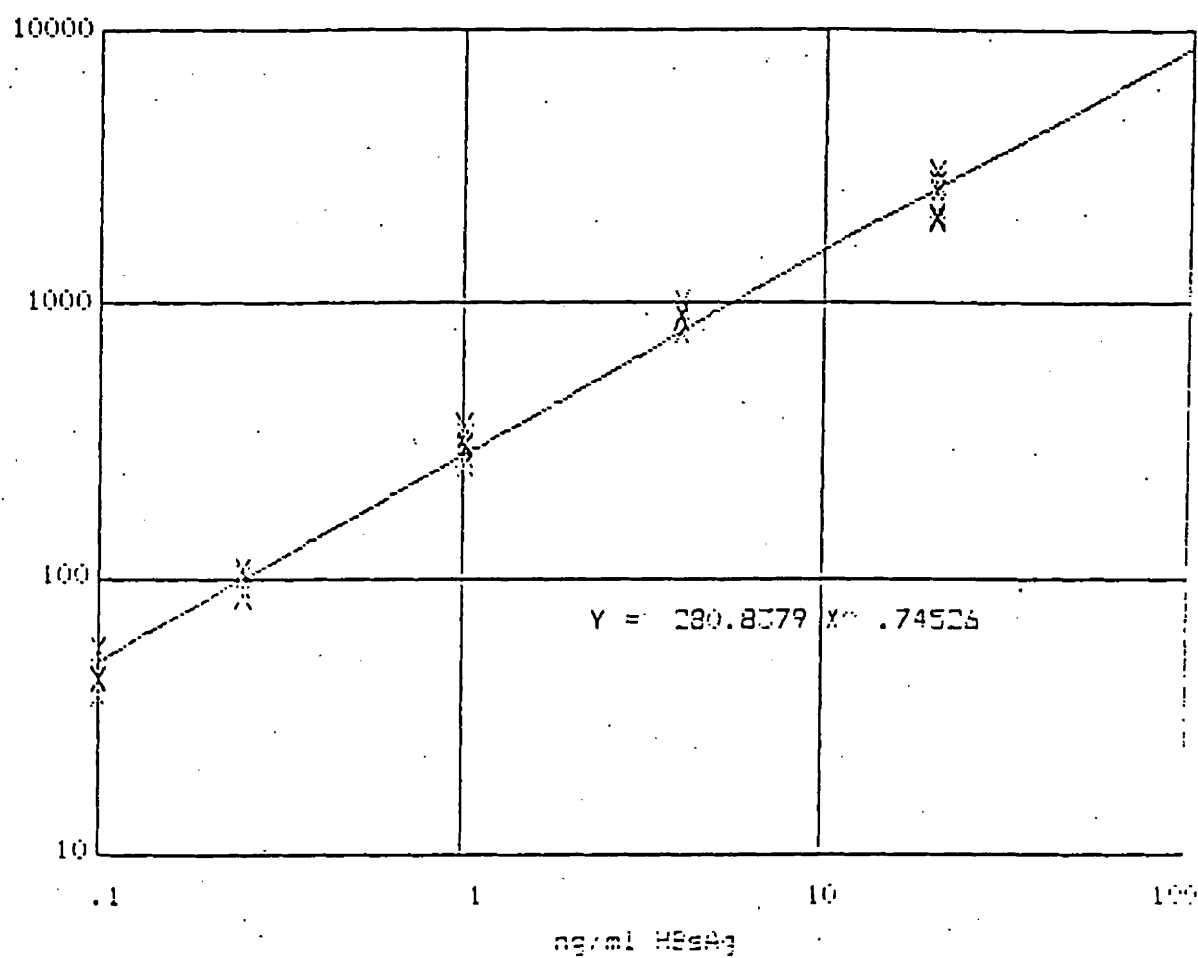


Figure 27.  
Calibration curve for RIA using BPL ng standards.

### Calculation of reproducibility of Bioelisa HBsAg.

All data in this study refers to results obtained by SPRIA and HA. However, during the progression of this study NLBTC changed from these two assays to an ELISA as their definitive test (March 1991). For completeness some data on this test has been included, since any further data in future studies will probably be obtained by this or a similar method.

In order to assess the reproducibility of this assay, it was used to assay several replicates of the BPL nanogram standards and the coefficients of variation were calculated.

### Results.

Figure 28: reproducibility of Bioelisa HBsAg.

ng/ml	0.1	0.25	0.5	1	4	10
n	16	16	8	8	8	10
mean OD	0.072	0.1196	0.179	0.313	1.015	1.997
SD	0.0039	0.005	0.0085	0.012	0.028	0.036
CV	5.44	4.28	4.73	3.977	2.78	1.79

Cut-off = 0.0928

### Observations.

The coefficients of variation show that in our hands this assay is certainly acceptable from the point of view of reproducibility. In this instance it exhibits a better coefficient of variation than either the RIA or the haemagglutination assay (see earlier data).

Many years of screening for HBsAg in donated blood at NLBTC has yielded a wealth of data. In section 4 I have attempted to summarise some of our data and compare it with some of the information provided by other workers in the field.

#### **SECTION 4: RESULTS AND ANALYSIS OF DATA.**

#### 4:1 Resource Material.

For the past eighteen years the North London Blood Transfusion Centre has maintained a library of samples collected from volunteer blood donors who have been found positive for hepatitis B surface antigen (HBsAg). By means of clinical, biochemical and serological analysis, these donors have been differentiated into those suffering from either an acute or a chronic HBV infection.

During the course of their infection, regular follow-up samples of HBsAg positive donors are obtained and examined for the relevant HBV markers in order to determine the nature and course of the infection. We are particularly interested in the rates of loss of surface antigen in the long term. This information helps us in the job of counselling donors and gives the donors confidence that their condition is being regularly monitored. It also reduces the chances of donors returning to donate on the normal donor panel. We are also able to discuss with them the relevance of the carrier state in terms of its effect on their sexual relationships, their offspring and their medical treatment, by monitoring levels of infectivity (HBeAg/Ab status). We also couple surface antigen testing with liver function tests and/or AFP as an index of carcinogenicity.

The results of all tests are collated on a computer database. This large reservoir of data provides us with material for long-term research in terms of the longevity of carriage of HBsAg, duration of infectivity and prognosis with regard to clearance of viral material and the likelihood of developing further complications such as primary hepatocellular carcinoma. By defining the characteristics of HBsAg positivity and by studying this in the context of the donor population of the centre and also nationally, it should be possible to be able to outline a spectrum of HBsAg reactivity and in particular to assess annually the range of rates of decline of HBsAg levels in different carriers. Therefore in future studies, if a group of carriers is identified as showing a rapid decline in HBsAg I will be able to examine any correlations between various parameters such as age, sex, ethnic origin, subtype or HBe status.

#### 4:2 Definition of population.

At NLBTC the records of all HBsAg positive donors followed-up are held on a computer database on a Data General Super MV 10000 using an in-house software package. The Antigen Donor Information System (ADIS) is designed to allow the storage, alteration and retrieval of information relating to hepatitis B surface antigen positive blood donors. An example of the stored information can be seen at figure 29. The information is divided into three different types:

Header information is information such as the donor's name, sex, date of birth, country of birth etc., i.e. items that occur only once for each donor, except that a donor may have more than one name.

Results information consists of the results of microbiological tests on samples from various bleed numbers and dates. Where a donor has only one header record he/she may have any number of results records.

Notes are simply textual records similar to medical notes.

There is also a data search facility written in to the software but this is very limited at present.

The regular updating of the donor records is done on a monthly basis and is the responsibility of the author.





#### 4:3 Analysis of donor population at NLBTC.

##### Definition of the background characteristics of the HBsAg positive population at the NLBTC.

As of 6th September 1988 the HBsAg-positive donor population on record at NLBTC was analysed as follows:

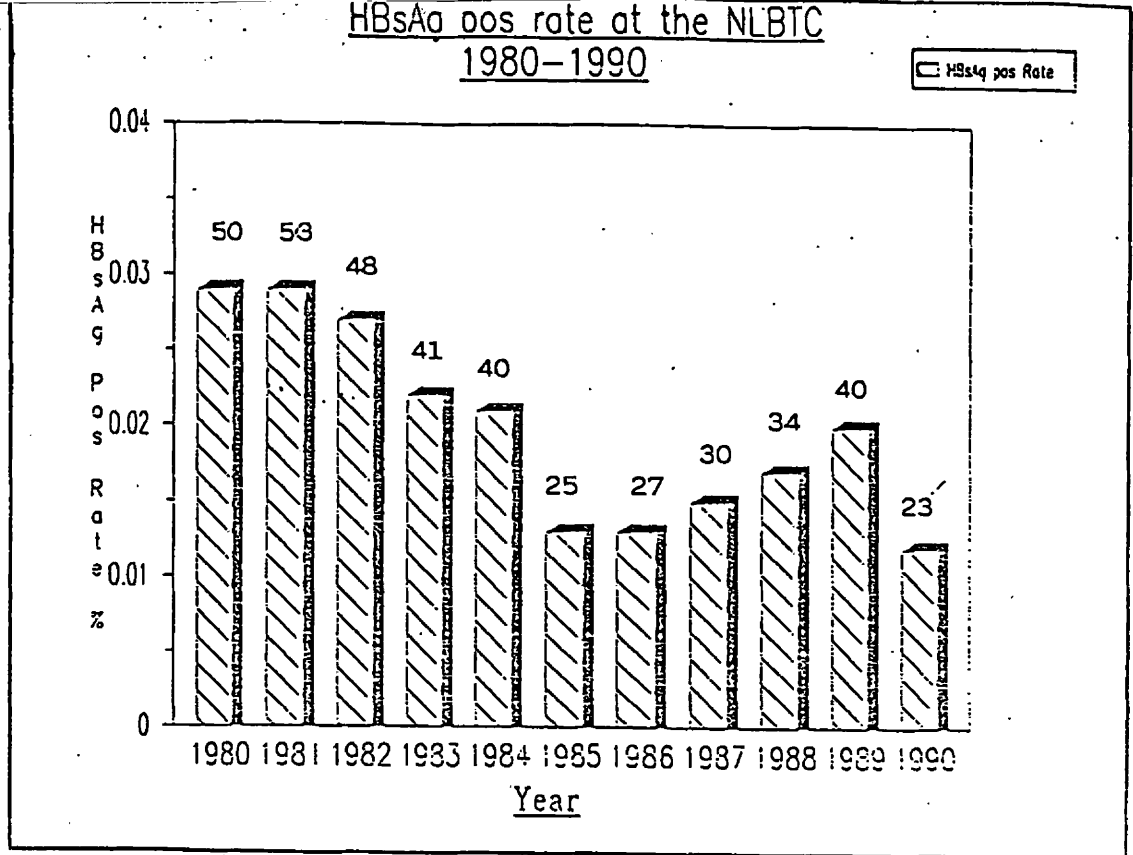
(i) The total number of HBsAg-positive donor records from 1971 was approximately 900 (i.e. 53 HBsAg positive donors per year overall).

(ii) From May 1974 detailed records have been kept and more detailed analysis will be based on these donors.

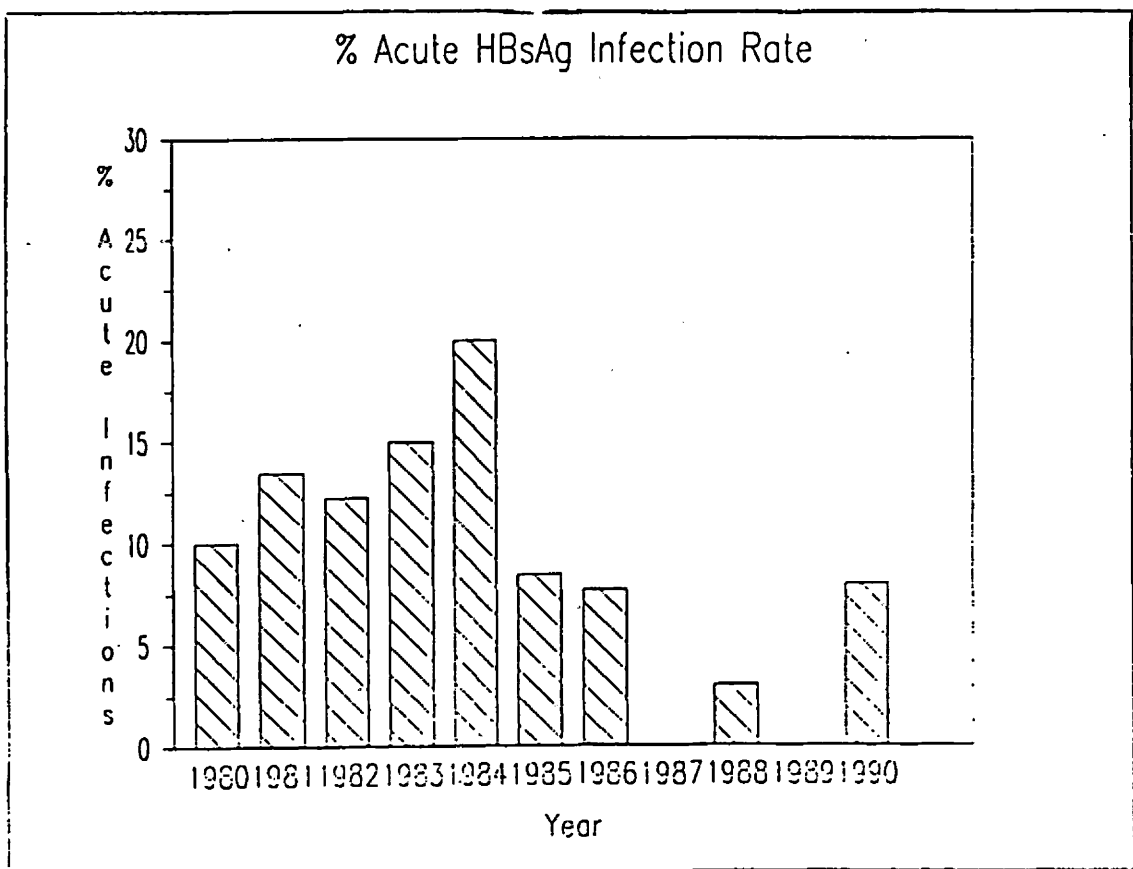
a) Surface antigen positive rate at NLBTC 1980-1990 (see figure 30.)

<u>Year</u>	<u>Total number.</u>	<u>HBsAg pos rate(%)</u>
1980	50	0.029
1981	53	0.029
1982	48	0.027
1983	41	0.022
1984	40	0.021
1985	25	0.013
1986	27	0.013
1987	30	0.015
1988	34	0.017
1989	40	0.020
1990	23	0.012

The HBsAg positive rate shown for 1980 and 1981 are representative of the rates for previous years.



**Figure 30.**  
Summary of the HBsAg positive rate at NLBTC 1980-1990.



**Figure 31.**  
HBsAg acute infection rate at NLBTC 1980-1990.

b) Acute infection rate as a percentage of all HBsAg positive donors at NLBTC per year. (See figure 31.)

<u>Year</u>	<u>Acute infections as a percentage of all HBsAg positive donors.</u>
1980	10%
1981	13.45%
1982	12.22%
1983	15%
1984	20%
1985	8.46%
1986	7.69%
1987	0.00%
1988	3%
1989	0.00%
1990	8%

c) HBsAg acute infection rate at NLBTC 1980-1990. (See figure 32)

<u>Year</u>	<u>Total No.</u>	<u>Acute infection rate(%).</u>
1980	6	0.0029
1981	7	0.0039
1982	6	0.0033
1983	6	0.0033
1984	8	0.0042
1985	2	0.0011
1986	2	0.0010
1987	0	0.0000
1988	1	0.00051
1989	0	0.0000
1990	3	0.0015

Figure 32 (a) shows the laboratory reports of acute infetions in the U.K. From the Communicable Disease reports from the Public Health

Laboratory Service. The sharp drop in infections seen at NLBTC was not seen nationally until 1988. This presumably reflects public awareness of HIV infection and the need to modify behaviour. The previously high levels around 1984 and 1985 were attributable to a large increase in intravenous drug abuse and a slight increase in homosexual promiscuity.

Laboratory reports of acute HBV in the U.K. 1980-1990.

1980	1007
1981	1197
1982	1237
1983	1274
1984	1937
1985	1771
1986	1313
1987	775
1988	636
1989	588
1990	564

HBsAg Acute  
Infection Rate (% of total donor pop)

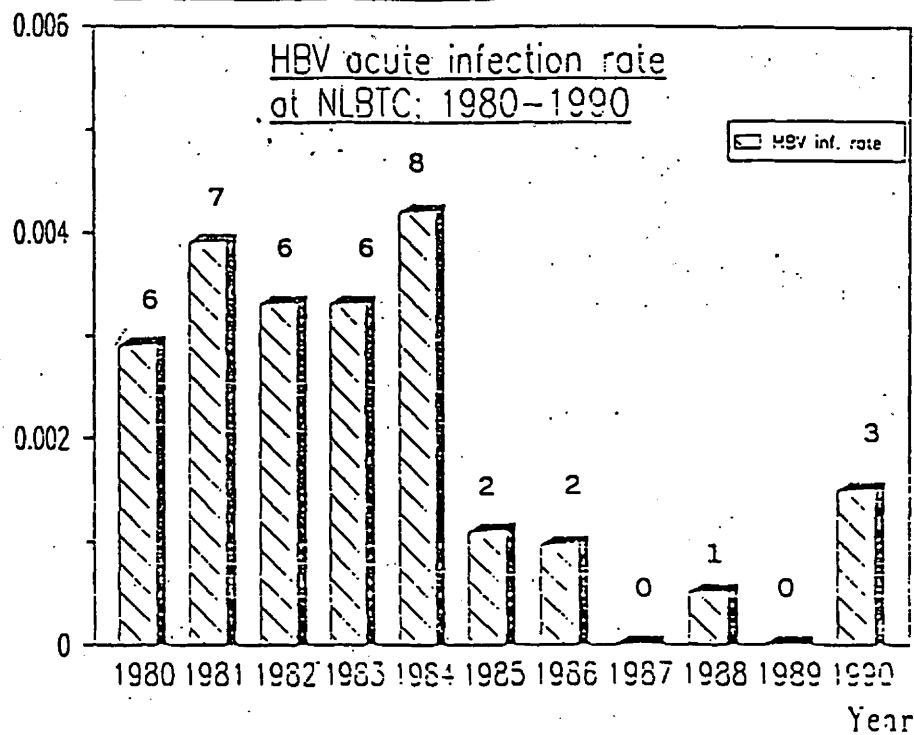


Figure 32.

HBsAg acute infection rate as a percentage of the total donor population at NLBTC 1980-1990.

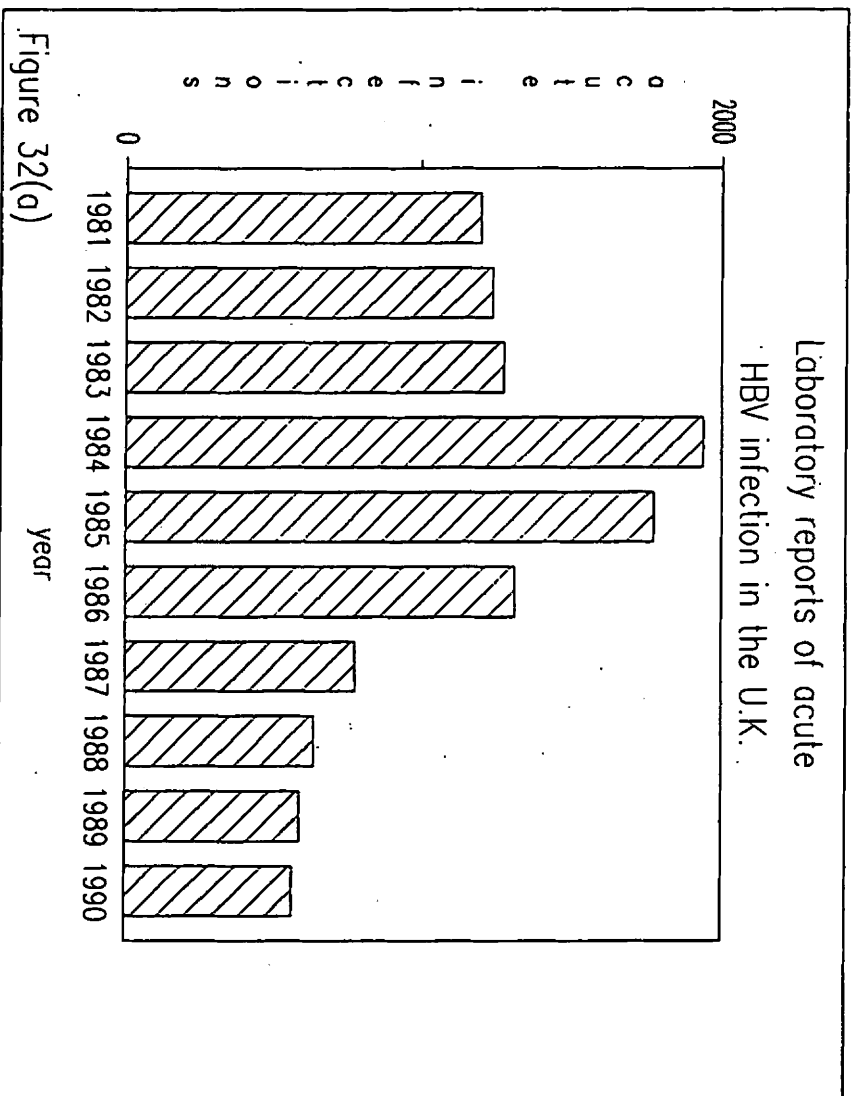


Figure 32(a)



Raw figures for population studied.

The population for close analysis was limited to those donors who fulfilled the following conditions:

1. There should be more than one donation or sample on record.
2. These donations/samples should be at least 3 months apart.

The resulting population was then analysed by a search of the data files:-

No of donors with selected criteria	= 332
No of acute infections	= 47
No of carriers	= 286
No of carriers HBeAg pos at detection	= 35

Table 3. Acute Infections.

Total number	47
Males	42 (89.4%)
Females	5 (10.6%)
Unknown gender	0 (0.0%)
New donors	14 (29.8%)
Old donors	27 (57.4%)
Previous donations at other centres	5 (10.6%)
Unknown donation history	1 (2.2%)
Born outside U.K.	7 (14.8%)
Born in U.K.	32 (68.1%)
Unknown country of birth	8 (17%)
Elevated LFT (>30)	19 (40.4%)

Table 4. Carriers.

Total number	286
>4 donations	151 (52.98%)
Males	202 (70.8%)
Females	77 (27%)
Unknown gender	7 (2.5%)
New donors	215 (75.4%)
Old donors	38 (13.3%)
Previous donations at other centres	26 (9.1%)
Unknown donation history	7 (2.5%)
Born outside U.K.	119 (41.8%)
Elevated LFT (>30)	128 (44.9%)

NB we do not have any data available on the risk status of the partners of our donors.

#### 4:4 Changes in HBe Status.

Changes in the HBe status were examined in detail. 35 (12.28%) of the 286 carriers studied were HBeAg positive at the time of pick-up, 27 (77.14%) of these 35 maintained their HBeAg positivity throughout their period of follow up. 8 (22.9%) of the 35 initially HBeAg positive went on to lose their HBe antigen and developed anti-HBe during the time we were able to follow them up.

At no time in the study did any carrier revert from anti-HBe to HBeAg.

It is also worth mentioning here that one of our donors who seroconverted from HBeAg to anti-HBe subsequently was found to have been undergoing Interferon therapy. It is interesting to note that he had shown changes in his liver function tests prior to seroconversion. The question remains, was his e seroconversion actually brought about by the Interferon therapy or just as the natural result of long-term carriage? It is noteworthy that we had documented his HBeAg status for 13 years prior to his e seroconversion.

Of the 286 carriers studied, 12 in all (4.2%), showed a change in HBe status; of these 12, 8 went from HBeAg to anti-HBe, 3 went from e neg, (neither detectable) to anti-HBe, and 1 went from anti-HBe to e neg during their follow-up period. The range of follow-up time for this group of 12 was 0.819 to 13.6 years and the mean follow-up time was 5.73 years. Of the 12

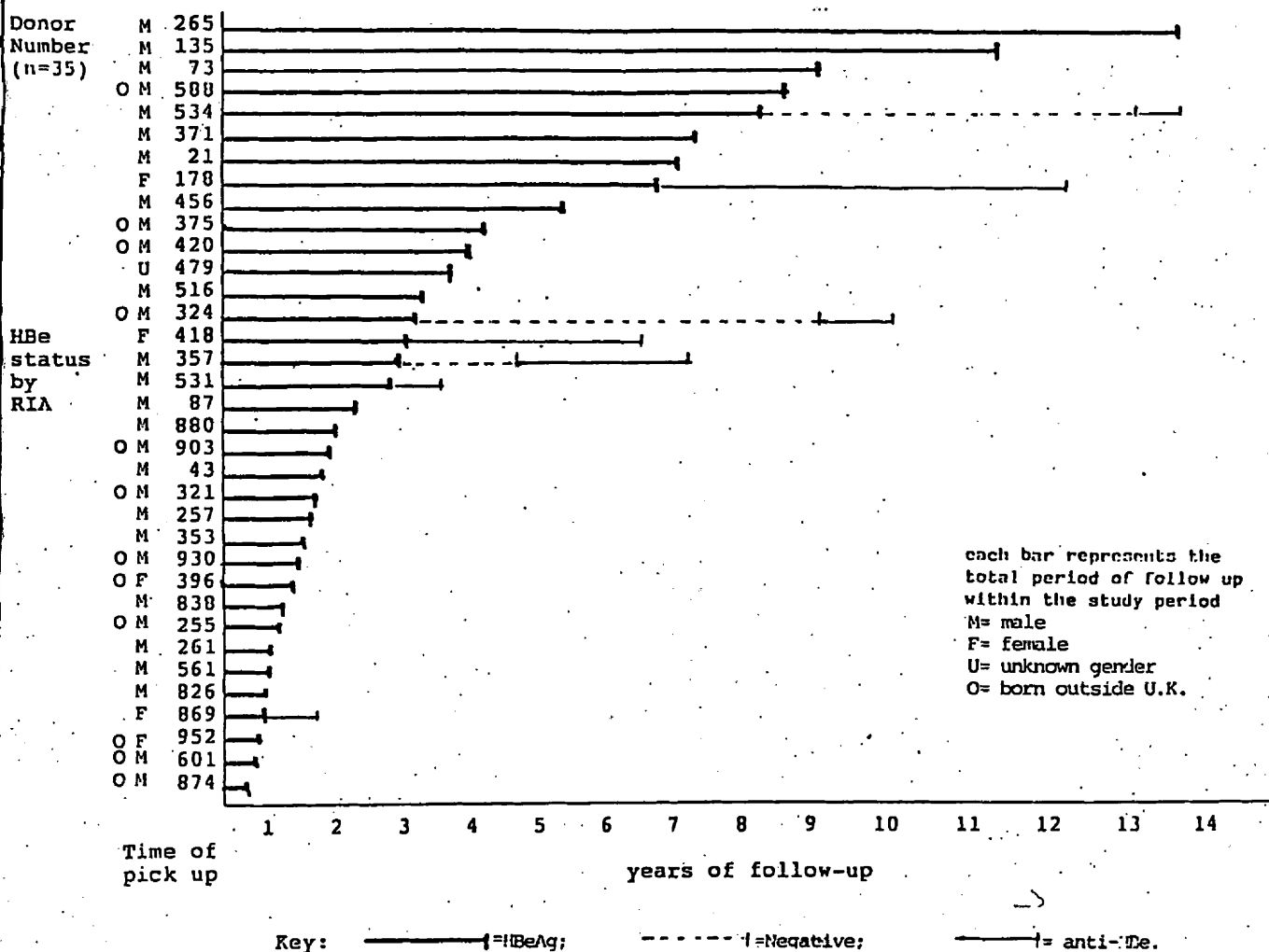
with HBe status changes 5 were of the ay subtype, whilst 6 were of the ad subtype, 1 was not subtyped. Also of these 12, 5 were born outside the U.K., 6 were born in the U.K. and 1 was of unknown country of origin. 8 of the 12 were males, 4 were female. 5 of them had stable titres whilst 7 showed a fall in titre (3 of them by a significant amount i.e. by a factor of three twofold dilutions or more.)

Figure 33.

Summary table of donors showing a change in HBe status.

HBe Status	Donor No.	Country of Orig.	Carrier Status	Years of follow up	subtype	no.of donations on record.	sex
Ag>Ab	178	UK	D	11.814	ad	19	F
Ag>Ab	324	O	D*	10.033	ad	8	M
Ag>Ab	357	UK	D*	8.008	ay	19	M
Ag>Ab	396	O	D*	0.942	ad	3	F
Ag>Ab	418	UK	S	7.211	ad	8	F
Ag>Ab	531	UK	D	3.030	ad	5	M
Ag>Ab	534	U	D	13.329	ay	32	M
Ag>Ab	869	UK	S	1.016	U	2	M
N>Ab	131	O	S	0.819	ay	3	M
N>Ab	243	UK	S	13.6	ad	12	M
N>Ab	677	O	S	6.334	ay	8	M
Ab>N	159	O	D	2.570	ay	3	F

key: D = HBsAg titre falling by less than 3 dilutions.  
D\* =HBsAg titre falling by greater than or equal to 3 dilutions.  
S = HBsAg titre stable. M = male . F = female.  
U = unknown gender.  
O = born outside U.K.  
Ag= HBeAg  
Ab= anti-HBe  
N= neither HBeAg or Ab detectable.



**Figure 34.**

HBe status by RIA throughout period of follow-up for those donors HBeAg positive at time of detection.

### Stability of HBeAg in population studied.

Out of a total of 286 carriers of HBsAg studied, 35 (12.28%) were positive for HBeAg at the time of detection. 29 were male, 5 were female, and one was of unknown gender. 27 of these 35 (77.1%) maintained their positivity for HBeAg throughout their period of follow-up. The mean period of follow-up for this group of 27 was 3.5 years. The range of follow-up time was 0.31 to 13.3 years. Of these 27 donors, 19 (70.4%) were subtyped and 17 (89.5%) of these subtyped donors were of the ad subtype, however ad is the prominent subtype in our donor population (59%) see Vox.Sang. 32:4-9 (1977). Of the remaining 2, one was ay and the other was ady. Also in this group of 27 donors, 17 (89.5%) showed an initial rise in LFT values, 4 (14.8%) showed a subsequent rise in LFTs and 6 (22.2%) remained within normal bounds for LFTs.

Titres of HBsAg for this group of 27 HBeAg positive donors ranged from 1 in 320 to 1 in 12,800. 17 (62.96%) of the 27 maintained a stable titre whilst 10 (37%) showed a drop in titre, 4 of whom showed a significant (3 wells or more) fall in titre during their period of follow-up.

Also in this group of 27 HBeAg positive donors 13 (48%) were born in the U.K. whilst 10 (37%) were born outside the U.K. The country of birth of 4 was unknown.

Figure 35. Table to summarise overall HBe status of eight donors who exhibited HBe seroconversion during follow-up.

Donor No	Duration of Markers.			total years of follow-up
	HBeAg	Negative	anti-HBe	
178	May 71- Oct 76		Oct 76- Feb 83	11.8
	5.42 yrs.		6.33yrs.	
324	Jul 78- Mar 82	Mar 82- Aug 87	Aug 87- May 88	10.03
	3.83yrs.	5.42yrs.	0.78yrs.	
357	Oct79- Dec 83	Dec 83- Aug 85	Aug 85- Feb 88	8.01
	3.16yrs.	1.66yrs	2.5yrs	
396	Mar 80- Mar81	Mar 81- May 81		0.94
	1yr.	0.16yrs		
418	Oct 77- Feb 81		Feb 81- Apr 84	7.2
	3.33yrs		3.16yrs	
531	Dec 78- Aug 81		Aug 81- Mar 82	3.03
	2.66yrs.		0.58yrs	
534	Jan 75- Apr 83	Apr 83- Dec 87	Dec 87- Jul 88	13.329
	8.25yrs.	4.66yrs.	0.58yrs.	
869	Aug 86- Mar 87		Mar 87- Dec 87	1.016
	0.58yrs.		0.75yrs	

(Dates accurate to the nearest month only.)



Correlation between raised LFT values and HBeAg seropositivity.

Of the 35 donors who were HBeAg positive at the time of detection, 24 (69%) exhibited elevated LFT values during the period of follow-up reflecting the association of HBeAg positivity and the presence of relatively large quantities of infectious virus. However seroconversion from HBeAg offers a better prognostic index since, out of the 8 donors who seroconverted from HBeAg during their time of follow-up, 5 were observed to reach a peak of ALT value prior to the point of seroconversion and in those who were monitored beyond the point of seroconversion the ALT levels eventually fell to within normal levels ( see table of normal values).

Figure 37. Table to show normal liver function test ranges.

total protein: 62-82 g/l

albumin: 36-52 g/l

AST: male : 4-45 iu/l

female: 4-30 iu/l

ALT: male : 4-52 iu/l

female: 4-29 iu/l

GGT male : 5-80 iu/l

female: 5-46 iu/l

Association between a rise in serum liver enzyme concentrations and the presence of HBeAg.

It has been suggested that the presence of HBeAg is closely associated with a slight rise in serum liver enzyme concentrations, a high HBsAg titre, and male sex. (Barbara, Mijovic et al. 1978) Examination of the population in this study would seem to corroborate this (see tables).

Figure 37.

Table of relationship between HBe and LFT.

HBe Status : LFT levels	Ab	Ab*	Ag	Ag*	N	N*	U	all
No	107	0	6	1	10	1	43	168
No*	19	1	4	1	1	0	0	26
R	25	0	16	5	5	1	10	62
R*	8	1	1	1	0	0	2	13
U	5	0	0	0	3	0	8	16
all	164	2	27	8	19	2	63	285

Key: Ab= anti-HBe positive.

Ab\*= initially anti-Hbe positive but result changed on subsequent samples.

Ag= HBeAg positive.

Ag\*=HBeAg positive at time of detection but result changed on subsequent samples.

N= neither HBeAg nor anti-HBe present.

N\*= initially neither marker detected but result changed on subsequent samples.

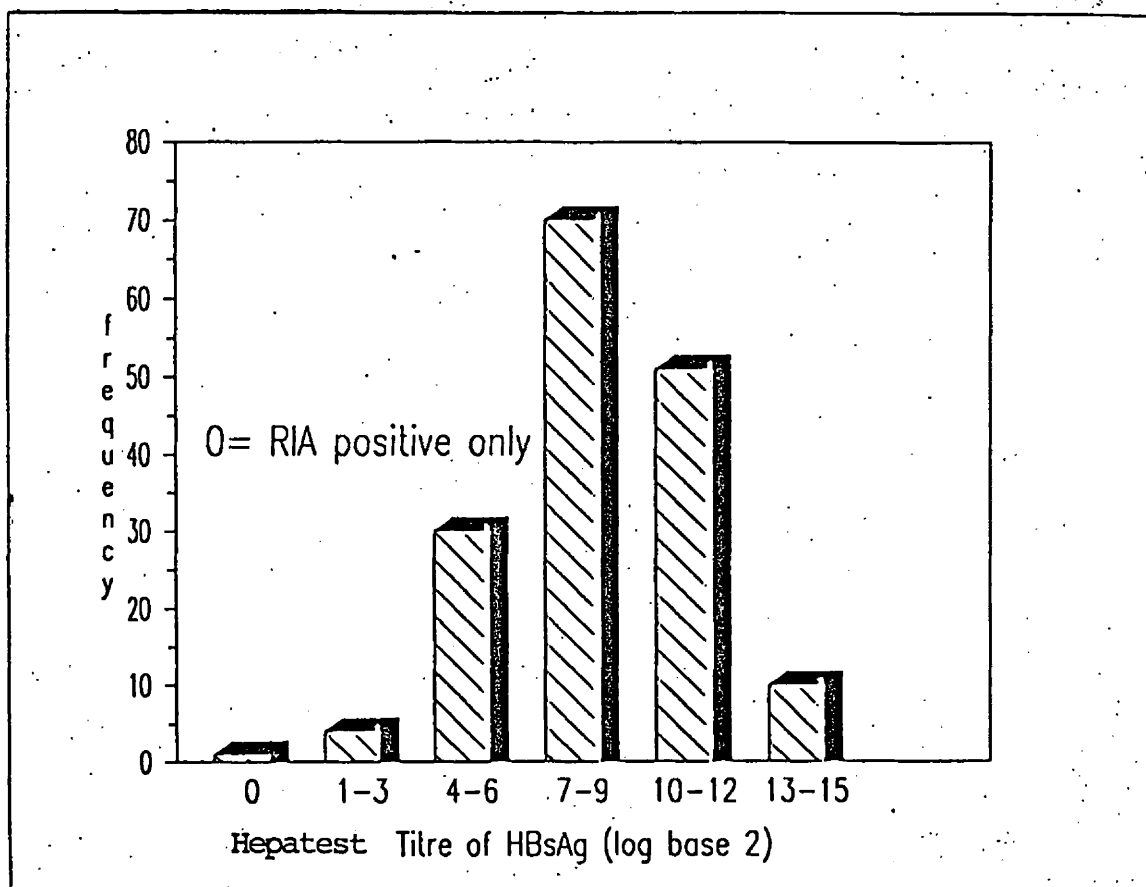
U= result unknown.

No=no rise in LFT values.

No\*=No initial rise in LFT values but a rise for subsequent samples.

R= rise in LFT values.

R\*= initial rise observed in LFT values but resumed normal levels for subsequent samples.



**Figure 38.**  
Distribution of titre of HBsAg at time of detection.

#### 4:5 Changes in titre of HBsAg.

Out of a total population of 286 carriers of HBsAg, 156 (54.7%) maintained a stable titre of HBsAg. 52 (18.2%) showed a significant fall in titre i.e. by three or more dilutions during the period of follow-up. 78 (27.4%) showed a fall of less than three dilutions during the period of follow-up.

Figure 39. Ranges of follow-up times in years for all carriers in study.

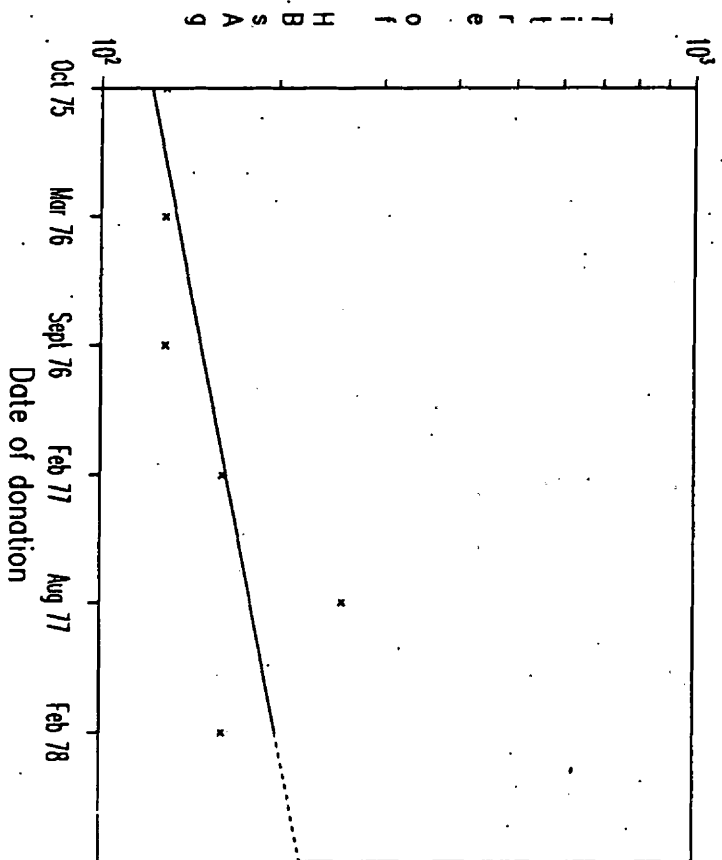
<u>Titres of HBsAg.</u>	<u>minimum</u>	<u>maximum</u>	<u>mean</u>
Stable titres	0.258	16.762	3.542
-----			
Falling <3 wells	0.277	13.710	4.946
-----			
Falling >=3 wells	0.942	16.997	8.465
-----			
Total	0.258	16.997	4.820
-----			

#### 4:6 Some typical donor HBsAg titre profiles with time.

On the following five pages some typical donor profiles are represented graphically.

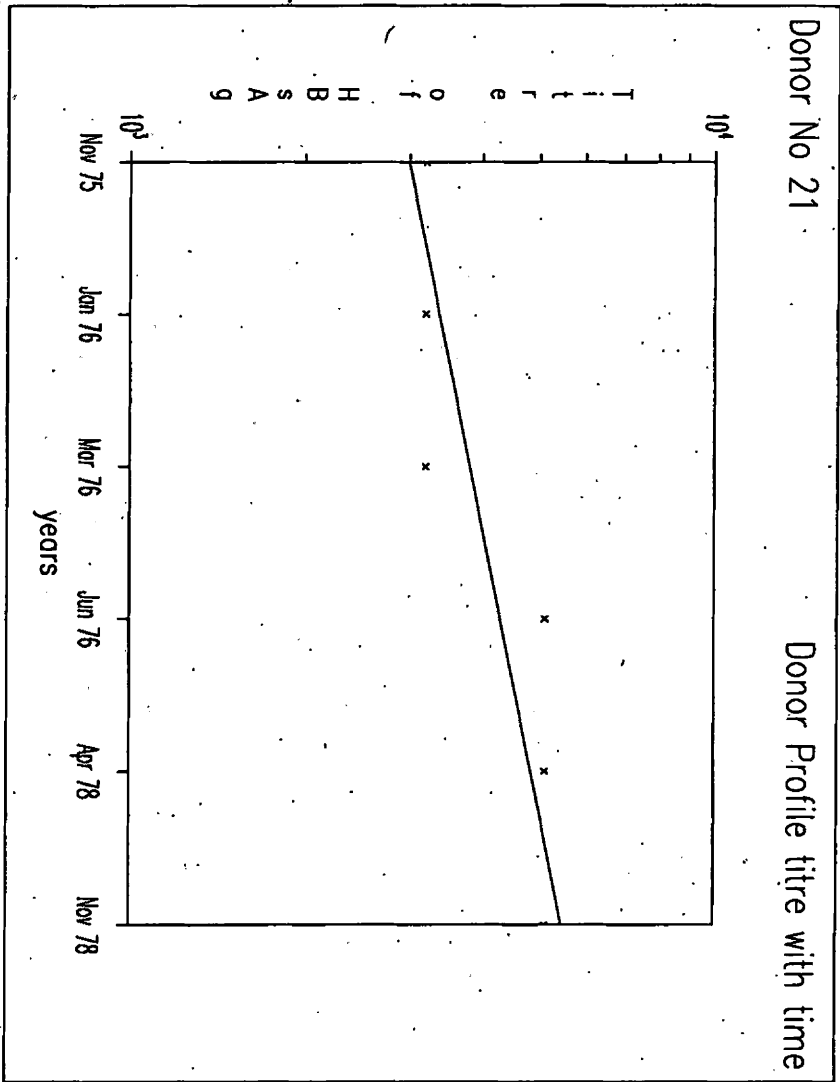
Donor No 12

Donor Profile titre with time.



Donor No 21

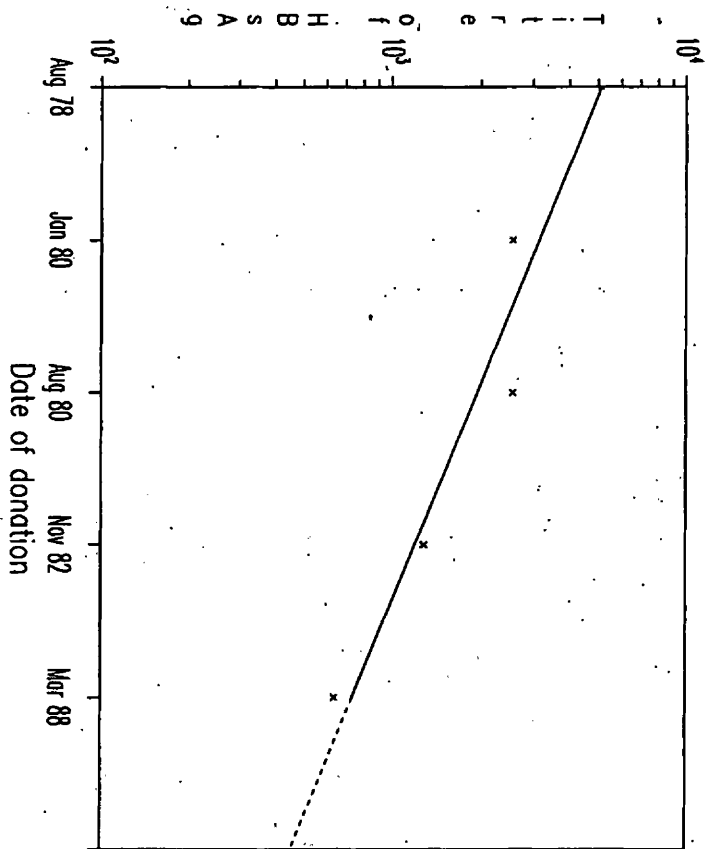
Donor Profile titre with time





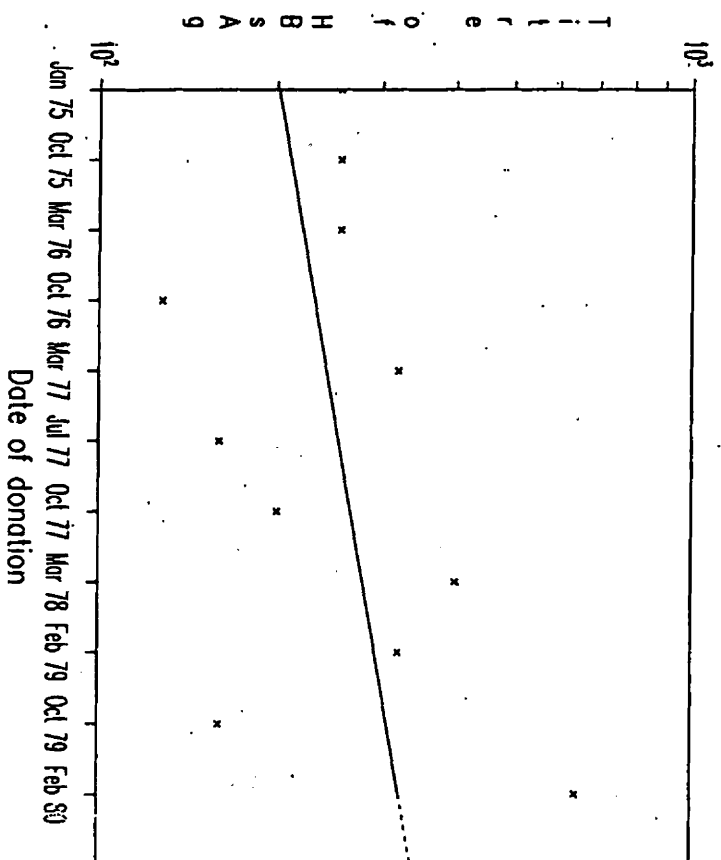
Donor No.66

Donor Profile titre with time



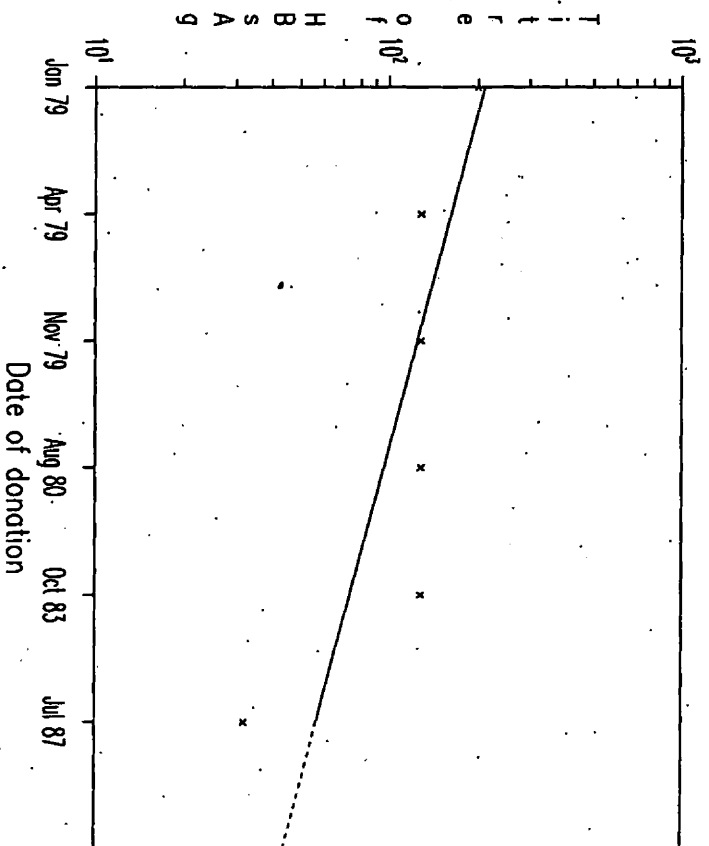
Donor No 228

Titre Profile with time.



Donor No. 821

Donor Profile titre with time



**DISCUSSION.**

## Discussion.

In this study I have attempted to illustrate the role of the hepatitis B virus and its associated markers in the context of blood transfusion microbiology. Currently virologists have access to a wide range of markers for hepatitis B virus infection and its associated disease. There is also much data available worldwide on the epidemiology of the virus and its transmission in various populations. However, even at this stage the picture is not totally complete. To gain an understanding of the carrier state in U.K. blood donors, we need an analysis of available data which can be put to both practical and epidemiological use. The information becomes particularly relevant in the field of donor counselling for reassuring the donor and forecasting what will happen to him in the future.

The data considered here contributes to our detailed understanding of the epidemiology of the hepatitis B virus in individual donors and also gives an insight into the situation of the British blood donor population as a whole. However, we are not seeing the situation as reflected in the 'normal' population, since blood donors at NLBTC are between the ages of 18 and 70 years of age and expected to be in good health. Potential donors who may fall into so-called "high-risk" groups such as intravenous drug users or homosexuals/bisexuals and their sexual

contacts and anyone who has had sex with a prostitute or anyone from a known high-risk area are asked not to give blood.

#### Investigation of test procedures.

It is of limited value to report only a "positive" or "negative" scoring of an HBsAg test for the safe release of blood and I wished to examine the various viral markers quantitatively in order to monitor changes in levels of HBsAg with time in our donor population. This study is the first stage in providing some data on this topic. In the first instance it was necessary to define the assays to be used, and to provide data on their precision, the spread of results and variations within assays on different days when using different batches of reagents and between different operators.

For example, in the case of the reverse passive haemagglutination assay, a series of end-point titrations were performed by two different operators on two different days using different batches of reagents. The results shown in figures 16-19 show that there was small variation between operators and batches: i.e. a CV between 5.1% and 8.7%. It was observed however, that a variation in titre of only one dilution resulted in a variation by a factor of one power of 2, since titres of virus obtained in this way are logarithmic. Although this is generally considered acceptable, it may result in rather a distorted picture in terms of the precision required when studying changes in

titre. The haemagglutination assay can be made more accurate if dilutions are made in smaller steps, however the advantage of an EIA or RIA is that there is a dose/response relationship over a given area of the calibration curve. I therefore realised that any future quantitation studies would need to be performed by the use of either an EIA or a SPRIA when direct reference can be made to a calibration curve of known standards in terms of nanograms of HBsAg per ml of serum. (See figure 27.)

#### Quantitation systems.

Theoretically there should be a simple linear relationship between counts per minute, optical density or fluorescence and antigen concentration. As this is the exception rather than the rule, where suitable reference preparations are available the concentrations can be extrapolated from a standard curve.

The basic assumption underlying the use of a standard to quantitate antigen in an immunoassay is that analytes in the standard and test specimens exhibit the same ability to bind at the solid-phase receptor sites. Ideally they should be identical in molecular configuration. Additionally the diluent which is selected for the reference preparation should closely resemble the sample. This can be difficult to achieve for all test samples due to normal variation in the constituents of serum. These differences can give rise to non-specific effects that alter the kinetics of the antigen-antibody reaction and can result in artifacts such

as non-specific binding or differential binding plateaux. However, it is not possible to tailor a reference preparation to each serum sample.

A parallelism study is useful for identifying and monitoring any changes in the assay performance. Ideally the standard and test specimens should exhibit parallel dilution curves. The practical importance of this is that if any test sample dilution is analysed, it will generate the same final dose estimate after interpolation from the standard curve, following correction for specimen dilution.

Heterologous interpolation is the method of choice where it is not possible to include a homologous reference serum for each specimen. A heterologous standard contains a known amount of analyte similar to the one being measured in the assay. The major requirement is that the dilution curves of the test sera and heterologous standard are parallel, to ensure that the interpolated dose is the same no matter what dilution is analysed. The results can be reported in weight per volume e.g. ng/ml.

In future studies, in order to examine the long-term kinetics of HBsAg carriage, I hope to initialise a quantitation system within the laboratory which will enable me to reassess the levels of HBsAg in our carrier population with a much greater degree of precision than that presently employed, i.e. the Wellcome Hepatest assay. I should also like to re-establish subtyping of our donors as this was abandoned sometime in the early eighties. I



could therefore examine our donor population and test whether or not the ad subtype is still predominant. This would also help to complete the data which is at present lacking. In addition I should like to examine tests for the detection of viral DNA such as PCR and DNA hybridisation assays as an aid to infectivity studies.

Data were initially collected for the assays which were currently in use for HBsAg testing at NLBTC at the time I began this study, that is for RPHA and for SPRIA. During the study changes were made in procedures and the current HBsAg assay is now EIA, therefore I include a brief characterisation of this assay in appendix 3. There is also an evaluation of this assay in the section on test evaluations. (See figure 29).

#### Preliminary analysis of accumulated data.

My main topic of investigation was an analysis of the accumulated data on the HBsAg-positive donor population at NLBTC where we have a unique collection of donor follow-up data gathered over a period of 18 or more years. This has received only cursory comment and analysis hitherto.

However there are potentially a large number of parameters for analysis, and for the purposes of this study it was necessary to concentrate on only some of these.

In September 1983 the first DHSS leaflet on AIDS in relation to blood donors was made available to blood donors. Throughout 1984 the level of AIDS education for

donors was stepped up. The aim was to specify those areas of "risk" behaviour for donors and to ask them not to attend if they had engaged in such behaviour. We predicted that this would also have an effect on the numbers of cases of HBV that we saw, and it did, (see figure 30.) presumably because 'at-risk' donors ceased to donate blood. Since HBV shares several routes of infection with HIV, (both are transmitted sexually and by intravenous drug abuse), donor "self-exclusion" due to AIDS education resulted in a dramatic fall in the rate of HBsAg positivity in donors subsequent to the introduction of measures to prevent HIV transmission by transfusion.

Prior to 1985, approximately half of our HBsAg-positive donors were from high-hepatitis-risk areas and half were native-born Britons. Most of these native-born HBsAg-positive donors were white, male and homosexual. Following the introduction of AIDS education, our HBsAg prevalence halved (see figure 30.). Overall, prior to 1984, 15% of all our HBsAg positive donors were undergoing an acute infection. Because half of all HBsAg positive donors were white, and most of the acute infections were in white donors, 30% of white, HBsAg-positive donors were undergoing an acute infection and a large proportion of the rest were HBeAg positive (i.e. recent) carriers. Subsequently, the number of acute HBV infections in our donors has dropped drastically, from 0.004% in 1984 to 0.00% in 1989 rising slightly to 0.0015% in 1990. (See figures 30 and 31).

If we did not differentiate between the acute and chronic infections then we would not see the full pattern of infection in the different donor populations. In the western world, acute HBV infections occur most commonly because of intravenous drug abuse and male homosexuality with some transmission from males to females; in contrast, the carrier state in high prevalence areas is most commonly seen as a result of vertical (mother to baby) transmission.

The majority of HBsAg positive donors are detected in the 'new donor' population because they have not been tested previously. If HBsAg is detected in a previously tested donor, it usually reflects acute infection, other possible reasons are changes to tests leading to increased sensitivity or very rarely, technical errors causing missed positives. From the data included in this study I can confirm the findings of others that infection is more likely to occur in males (Gerety 1985) as 89.4% of acute infections and 70.8% of carriers in my population sample were male. I can also confirm that carriers are more likely to come from tropical or mediterranean areas, (Barbara 1983) as 41.8% of carriers were born outside the U.K. and only 14.8% of acute infections were born outside the U.K.

Donors from high-hepatitis-risk areas constitute less than 2% of our donor population and therefore exert a disproportionate effect on our HBsAg positive rate with respect to their small numbers. Most of the other published work refers to studies done before the advent of HIV infection; post-AIDS, if anything, this effect is

heightened because of the self exclusion of homosexuals and intravenous drug abusers and also because of our encouragement to the ethnic minorities to donate blood to serve our current patient population, which includes large numbers of these ethnic minorities needing supplies of the rarer (to the U.K.) blood groups.

### Duration of infection and the carrier state.

When counselling donors who are HBeAg positive it is necessary for their peace of mind to be able to predict to some extent how long they are going to be infectious because of the need to try to prevent transmission from mother to child, sexual transmission and also accidental transmission. For this reason a major part of my work has been the analysis of HBe antigen and antibody rates. Previous findings on percentage HBeAg to anti-HBe seroconversion rates cite a 5%-25% per annum conversion rate (Lok et al. 1987.). The data for our 35 HBeAg-positive donors was subjected to a Kaplan-Meier analysis which produced an estimated lower quartile time to seroconversion of 3.83 years and a median seroconversion time of 8.25 years. (The upper quartile could not be estimated due to there being less information available). This corresponds to an average rate of 6.5% per annum for the first 25% to seroconvert and 5.7% for the next 25%.

Previous estimates have not been made on a comparable basis so it is difficult to compare other studies with our own, but previous data range from 3% per annum (Viola et al. 1981 U.K.) to 21% per annum (Hoofnagle et al. 1981 U.S.A.). Weller et al. 1985(U.K.) state a seroconversion rate of 10% per annum using only the HBeAg-positive time of follow-up and not the HBeAg-negative period that followed loss of HBeAg which falsely lowers the true spontaneous rate of loss of HBeAg. Our figure of 5-6% per annum lies at the lowest end of the scale probably due to the fact that we

followed our donors for a much longer period of time and also the fact that our donor population have been pre-selected as fit, healthy individuals between the ages of 18 and 65 years.

I wanted to undertake a detailed analysis of the length of time of HBeAg positivity because of its significance with respect to infectivity , i.e. vertical transmission, sexual transmission, and "needlestick" (small volume) transmission, and also as a prognostic indicator of clinical sequelae, as HBeAg is associated with raised ALT levels in chronic liver disease. This is of crucial significance for the effective counselling of donors confirmed to be HBsAg-positive. When a donor is confirmed to be HBsAg-positive, we feel it is our duty to provide them with the fullest advice possible on the significance of our findings and to discuss the significance of these findings for the future of the donor. My study has provided a sound basis for counselling because it includes the detailed follow-up over a long period of time of a large number of cases.

### Markers of infectivity.

HBeAg is commonly considered the most indicative marker of infectivity because of its high level of correlation with the presence of replicating virus material. At present we do not quantitate this marker routinely in our laboratory. However with the RIA assay which we use, this could be a possibility. However it is now known that HBeAg and anti-HBe are not totally reliable as parameters of viraemia. (Bonino et.al. 1981 and 1986.) As many as 10-15% of viraemic anti-HBe-positive HBsAg carriers can have more than  $10^5$  HBV genomes per ml of serum.

A most important marker of viral replication is viral DNA. This can be assayed by either PCR or DNA hybridisation (see page 90). Detection of HBV DNA by spot hybridisation correlates well with infectivity and the number of genomes present. However I am led to conjecture here that if ultimate sensitivity of viral replication were required, perhaps we should be attempting PCR amplification of the RNA pregenome which is transcribed to DNA by reverse transcriptase during viral replication. Amplification of this RNA would truly reflect active viral replication and not simply the presence of non-replicating genomic material.

Another marker of infectivity is DNA polymerase. We do not test for this marker at present as there are more important

markers of infectivity for which sensitive screening tests are available.

#### Infectivity in blood donors.

In carriers (defined formally as carriage of HBsAg for 6 months or more), of the 12.28% who were HBeAg positive, 77.14% maintained their HBsAg positivity throughout their follow up period (mean 4.25 years). 22.9% of those donors found positive for HBeAg, subsequently went on to develop anti-HBe during the course of their period of follow-up, giving a 5% per annum seroconversion rate. Since our HBeAg longevity data is based on long-term, detailed follow-up on a relatively large number of donors it is likely to be accurate. We have seen that there is variation in HBeAg longevity in carriers but close analysis concurs with that of other researchers studying patients in Sexually Transmitted Disease clinics etc. That is, that some carriers maintain HBeAg for very long periods of time ( see figure 34. donor no. 265, still maintaining HBeAg after 14 years of follow-up) and that on average 5-25% per annum seroconvert to anti-HBe.

#### Stability of titre.

The longevity of surface antigen carriage is to be the subject of future research, but our preliminary data showed that 54.7% maintained stability of titre throughout the period of follow-up, with 18.2% falling by three dilutions



or more and 27.4% falling by less than three dilutions during their period of follow-up (mean 4.82 yrs.).

Many centres still persist in calling HBsAg positive donors "carriers" without a full characterisation of those markers which confirm the carrier state, that is anti-HBc IgM, and parallel titrations of HBsAg in the serum, often these are not real carriers.

The association between a rise in liver function test levels and HBeAg positivity demonstrated by Barbara, Mijovic et al. (1978). was corroborated by continual follow-up data in my study in that out of 27 HBeAg-positive donors, an initial rise in LFT levels was observed in 17 (62.96%) and a subsequent rise in 4 (14.8%), whilst normal LFTs were observed in 107 out of 164 anti-HBe positive carriers (65%). Also, in the 8 donors who eventually seroconverted from HBeAg, elevated LFT values were seen in 5 of these, with normal LFT values eventually restored in the three who were monitored continually for some time following their seroconversion. Therefore the conclusion drawn from this data is that approximately 5% per annum of donors positive for HBeAg will go on to seroconvert to anti-HBe with the associated reduced infectivity risks and better prognosis for their long-term health and well-being. This information can give considerable reassurance to donors during counselling.

### A glimpse of the future.

Data collected at one centre over many years can have considerable potential value for the epidemiologist. Take for example the recently-cloned hepatitis C virus; unlike HBV, which has been studied since the 1960s, our understanding of HCV is in an early phase, epidemiologically speaking. At this stage we do not know which antibody-positive donors are really infectious and so we have to assume all are. We do not know if there are different levels of infectivity and we do not know how the virus spreads in nature. However with HBV we now know that there is less chance of natural infectivity from blood or sexual contact from an anti-HBe positive donor than with blood from a donor positive for HBeAg. We know that HBV spreads via blood and semen. This results in potential transmission by sexual contact and intravenous drug abuse and also in certain institutions such as mental hospitals where residents may cause each other subcutaneous injuries by scratching or biting.

Clearly, the more complete our understanding of the mechanisms of transmission and the duration of infectivity, the greater our chance of reducing the risks. The field of transfusion microbiology is constantly changing and rapidly responding to changing requirements (see section 2). During the course of this study at NLBTC we have been faced with the evaluation and implementation of a new and different form of assay for the routine testing of blood donations for the HBV marker, hepatitis B surface antigen, we have

now changed from a SPRIA to an EIA assay. Today we also face the challenges of adjusting to laboratory automation and information transfer. Detailed study of epidemiology of HBV markers should help us to optimise the strategy used to provide a safer product. Soon we may be asked to begin testing for another HBV marker, anti-HBc, and the need to test routinely for this marker is currently under debate. If implemented, this assay will provide an even greater level of sensitivity in HBV testing, since donors with anti-HBc as the only marker of infectivity (with subliminal levels of HBsAg) have been shown to transmit post-transfusion HBV, which can be fatal in the immunosuppressed patient.

My study illustrates the range of data that can be accumulated by follow-up of donors found to be carrying HBsAg. It serves as a base line for further work to examine such donors in more detail, thus providing a better overall understanding of the carrier phenomenon. The same approach would be useful to the follow-up of donors with other persistent infections with viruses such as HCV.

**APPENDICES: SUMMARIES OF PROTOCOLS OF ASSAYS USED IN  
PROVIDING THE EXPERIMENTAL DATA.**

Appendix 1: Solid Phase Immunoradiometric Assay for  
Hepatitis B Surface Antigen Detection.

Reagents used in this study are supplied by the Blood Products Laboratory (BPL) Elstree.

(a) Anti-HBs Immunoglobulin for Coating of  
Microwells.

High-titre anti-HBs serum from immunised horses is heat-inactivated at 56°C for 30 mins. and then absorbed for anti-human antibodies by passage through affinity columns. The globulins are precipitated in 40% saturated  $(\text{NH}_4)_2\text{SO}_4$  at 4°C, redissolved in Tris/Az containing 0.9% NaCl to the original volume (Tris/Sal/Az) and dialysed against Tris/Sal/Az.

(b) <sup>125</sup>I-labelled anti-HBs.

Purified anti-HBs from heat-inactivated goat anti-serum is iodinated (Cameron et al. 1980).

The labelled antibody is diluted to a count of 25 nano-curies (nCi) per 0.1 ml in Tris/Az containing 10% normal human serum (NHS), 6% normal horse serum and 4% normal goat serum. The diluted labelled antibody gives a radioactive count of approximately 40,000 c.p.m. per 0.1 ml.

(c) Positive Control Serum.

Positive control serum is obtained from a single high titre HBsAg positive donor and diluted to levels of 20ng/ml, 4ng/ml, 1ng/ml, 0.25ng/ml, and 0.1ng/ml in NHS containing 0.1% sodium azide.

(d) Negative Control Serum.

Negative control serum is from a pool of normal human serum found negative by radioimmunoassay for HBsAg, anti-HBs and anti-HBc, and contains 0.1% sodium azide.

(e) 0.02M Tris/HCL Buffer with 0.1% Sodium Azide.

This is prepared in the laboratory as follows:

Dissolve :	Tris(hydroxymethyl)methylamine	12.114g
	in 1 litre deionised water	
Add	: Sodium Azide	5.0g
	5M HCL	13.75ml

The final volume is adjusted to 5 litres with deionised water. The final pH should be 7.6., this is adjusted by the addition of HCL.

The principle of this assay is that of the labelled antibody "sandwich" type of immunoassay illustrated in Section 3, figure 12 (E).

#### Test Procedure.

0.1 ml of serum or plasma is incubated in the antibody coated wells in a waterbath at 45°C for 90 mins. The wells are then washed five times in de-ionised water. 0.1 ml of radiolabelled antibody is then added to each well. The wells are incubated as before in the 40°C waterbath for a further 90 mins. They are then re-washed as before and counted in a gamma counter. The counts per minute can be compared with a set of known standard negatives from BPL, but calculation of the cut-off is performed by computer.

A mean and standard deviation is calculated for all wells with a count less than 100 c.p.m. All results within the mean plus or minus 3 standard deviations are used to calculate a new mean and standard deviation. A result is deemed positive if it is greater than the new mean plus 3 standard deviations.

If for any reason the computer is non-functional the cut-off can be calculated from the raw data by taking the mean of a random sample of negatives and multiplying this by 1.5.

## Appendix 2: Reverse Passive Haemagglutination Assay (RPHA)

The principle of the test is that the reagent red blood cells have been treated with tannic acid, purified horse anti-HBs can then attach to produce "sensitised" cells which will agglutinate in the presence of HBsAg. So that non-specific reactions may be detected, "control" cells coated with pre-immune normal horse immunoglobulin are also used. Both test and control cells have been treated with formalin and freeze-dried for stability on storage.

### Reagents.

#### (a) Test Cells.

These consist of a freeze-dried 1% suspension of aldehyde treated, tanned turkey red blood cells coated with purified horse anti-HBsAg in phosphate buffered saline (pH 7.2) containing 5% sucrose, 1.5% normal rabbit serum and 0.01% thiomersal.

#### (b) Control Cells.

These consist of a freeze-dried 1% suspension of aldehyde treated, tanned turkey red blood cells coated with normal horse globulin, in phosphate buffered saline (pH 7.2) containing 5% sucrose 1.5% normal rabbit serum and 0.01% thiomersal.



(c) Diluent Buffer.

Phosphate buffered saline (pH 7.2) containing normal human serum, normal turkey serum and 0.1% sodium azide. The volumes of normal sera are adjusted to give the optimal results for each batch of sensitised cells hence the need to use a complete set of matched reagents.

(d) Positive Control.

Diluted human serum inactivated by heating at 60°C for at least 10 hours. When tested at a 1:8 dilution, the positive control should give a clear agglutinated pattern with the test cells. When fully titrated the end-point should be not less than 1:32 against test cells, while the control cells should show unagglutinated patterns at all dilutions. Failure to comply with all of the above conditions indicates deterioration of either test or control cells or contamination of diluent buffer.

(e) Negative Control.

Normal human serum. No agglutination should be observed with either test or control cells. Failure to comply is indicative of deterioration of test or control cells or contamination of diluent buffer. Both negative and positive control sera contain 0.1% sodium azide.

## Test Procedure.

### Quantitative Test.

#### Method.

Using a standard 25 microlitre dropper, one drop (0.025 ml) of diluent is placed in each well of a "U"-well microtitre plate. Two rows of wells are used for each sample to be titrated. The first row is used to demonstrate the reaction with the test cells, and the second to demonstrate the reaction with the control cells.

0.025 ml of serum is added to the first well of both test and control rows with a micropipette or microdiluter. Serum and diluent are carefully mixed and 0.025 ml of the mixture is transferred to the next well of each row, giving a dilution of 1:4. This procedure is repeated for each well of the two rows to give serial dilutions from 1:2 to 1:256.

Using a 0.025 ml dropper, one drop of reconstituted test cells is added to each well of the test row, starting with the 1:2 dilution.

One drop of reconstituted control cells is similarly added to the control row. The plate is then tapped on all four sides to mix the reagents thoroughly.

The plate is then covered with a lid and allowed to settle on a flat surface away from vibration or direct sunlight.

### Interpretation of results.

The end-point is taken as the highest dilution showing definite signs of agglutination, judged by the size and appearance of the ring.

A positive result for HBsAg is indicated by the presence of at least a fourfold greater titre in the test row than in the control row. Agglutination in the control row to within one well of the test row indicates a non-specific reaction. Full confirmation should always be obtained in haemagglutination test by the use of specific neutralisation tests however, or by the use of alternative procedures of equal or greater sensitivity such as SPRIA or ELISA techniques.

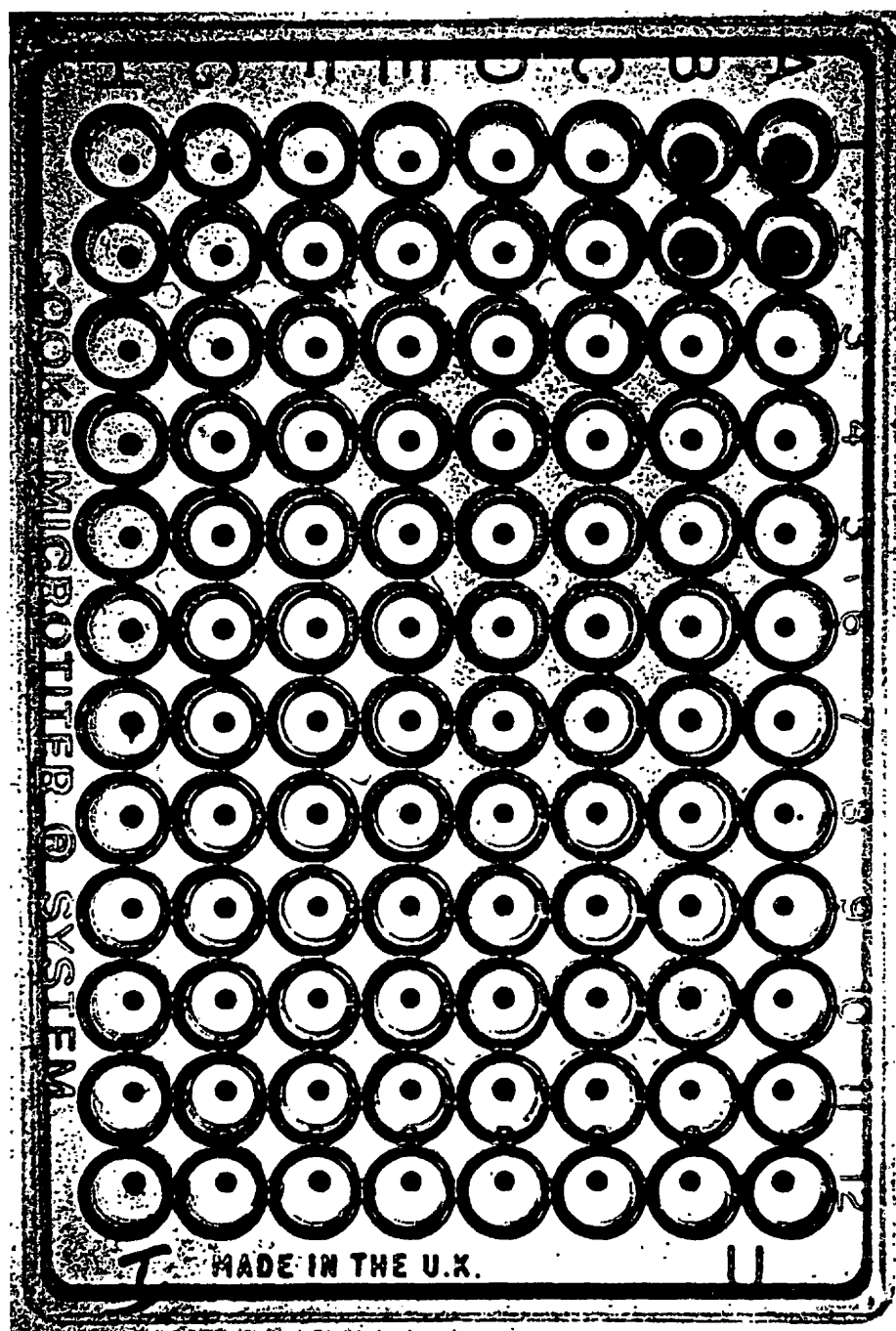


Plate 5 . Microtitration plate showing Wellcome Hepatest  
with positive control wells A1, B1, A2, B2.

### Appendix 3: Bioelisa EIA for HBsAg.

The reagents are supplied by Launch Diagnostics and are manufactured by Biokit S.A. in Barcelona, Spain.

At NLBTC we use a shortened protocol involving the use of a shaker incubator for the sample and conjugate stages of incubation to ensure adequate sensitivity whilst keeping incubation time to a minimum.

Bioelisa is a direct immunoenzymatic method of the "sandwich" type, in which guinea-pig anti-HBs coated on to microtitre plate wells acts as the capture antibody. Goat anti-HBs marked with peroxidase serves as the conjugate.

#### Test procedure.

0.1 ml of the serum samples and positive and negative controls are incubated in the antibody-coated wells. If the sample contains HBsAg, the antigen will bind to the antibody on the plate. After washing to eliminate any unbound material, 0.1 ml of goat anti-HBs conjugate is added to the wells and allowed to react with the antigen-antibody complex formed in the first incubation. After this second incubation and subsequent washing an enzyme substrate (hydrogen peroxide) solution containing TMB chromogen is added. This substrate (hydrogen peroxide) will develop colour if the sample is HBsAg positive. The intensity of the colour is proportional to the amount of HBsAg in the test specimens. The reaction is stopped by the

addition of 0.1 ml of 2M  $\text{H}_2\text{SO}_4$ . The colour intensity is read in a spectrophotometer at dual wavelengths of 450 and 620 nm respectively.

In our hands this shaking incubation procedure is sensitive to a level of approximately 0.2 ng of HBsAg per ml of serum.

#### Appendix 4: Specific Neutralisation Techniques.

##### (i) SPRIA.

Samples giving a repeatable positive screen test can be confirmed by performing a specific neutralisation test using normal and immune human, rabbit and horse sera.

The test is performed by setting up six tests pairing normal and immune sera in the proportions 0.1 ml test serum with 0.02 ml normal or immune serum in the same well and then proceed as for the normal screen test.

Specific neutralisation is confirmed when the count for the immune serum is at least half the count for the normal serum. With a very strong positive serum it may be necessary to perform the neutralisation test on a suitable dilution to avoid the immune serum being overpowered by large amounts of viral antigen. (Cameron, C.H. and Briggs, M. J. Virol. Meth. 1980 1 113-116)

This method can also be used to attempt specific neutralisation using the Bioelisa HBsAg EIA assay except that the origin of the immune sera is human, from two high-titre anti-HBs positive donors, rather than horse or rabbit as for SPRIA. Reduction in optical density of 50% or more indicates a successful neutralisation. If required the anti-sera may be allowed to react for 20 mins in a test tube with the reactive sera before the test is performed.

(ii) RPHA

When a HBsAg titre has been obtained by the standard Hepatest method a specific neutralisation test can be carried out to confirm the positive result.

The test is performed in triplicate on a suitable dilution of the serum. Standard titrations are made in three rows of the microtitre plate and 0.025 ml of normal and immune horse serum are added to the three wells before the known end-point, test cells are added to the first two rows and control cells to the third row :

Neutralisation Well	1	2	3	
Row 1	-	-	-	Immune serum + test cells
Row 2	+	+	+	Normal serum + test cells
Row 3	-	-	-	Normal serum + control cells

Neutralisation is confirmed if the above pattern is seen where the positive reaction is neutralised by the immune serum but the positive reaction is still observed with the normal serum.



Appendix 5: Monoclonal Radio-Immunoassay (RIA) for  
HBeAg/Ab.

The reagents are supplied by the Department of Virology, Middlesex Hospital Medical School and are used routinely at NLBTC on all HBsAg-positive serum samples.

(i) The principle of the test is similar to that described for SPRIA for HBsAg except that HBeAg is added to the antibody-coated wells on top of the test serum so that anti-HBe-positive serum will neutralise the antigen resulting in detection of low levels of bound radiolabel, HBeAg-positive serum will yield very high counts-per-minute, whilst the negative range, where neither antigen nor antibody are detectable, lies somewhere in the middle of the two. Test results are compared with a series of known HBeAg, anti-HBe and negative control sera included on each test plate.

(ii) Reagents.

(a) Anti-HBe Immunoglobulin-Coated Microwells.

"Removastrip" microwells are coated over three days with a mixture of monoclonal e6 and e9 gammaglobulin precipitates, diluted 1 in 2000 in 0.02M Tris/Azide to give a total globulin concentration of 1 in 1000. The wells are then quenched with 0.2% bovine serum albumin (BSA) and stored at 4°C.

(b) Controls.

Positive control serum consists of known HBeAg-positive donor serum diluted to 1 in 1000 in normal human serum (NHS). Negative control serum consists of a pool of known anti-HBe-positive sera diluted to 1 in 3000 in NHS. Negative control serum consists of pooled NHS.

(c) Radiolabel.

This consists of  $^{125}\text{I}$ -labelled e2 gammaglobulin diluted in "e diluent" to give approximately 400,000 counts-per-10 minutes.

(d) "e Diluent".

This is made in the laboratory as follows:

Bovine serum albumin.....	2%
Normal human serum.....	4%
in 0.02M Tris	

Test Procedure.

0.05 ml of sample and controls are added to the coated wells 0.05 ml of HBeAg control is then added to all wells including both samples and controls. These wells are incubated overnight at room temperature with a cover over the plate. The wells are washed five times in de-ionised

water and then 0.1 ml of radiolabelled antibody is added to each one. The wells are then incubated for 2-4 hours at room temperature and then washed again in de-ionised water. The wells are then counted in a gamma counter.

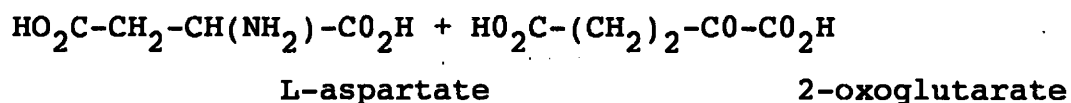
The results are then evaluated by a computer. The "cut-offs" are calculated as follows; any result greater than or equal to the mean of the four HBeAg controls is HBeAg positive, any result equal to or less than the mean of the four anti-HBe controls is anti-HBe positive. Any result falling between the means of both these controls is said to have neither HBeAg nor anti-HBe detectable.

Appendix 6: Estimation of Serum alanine  
aminotransferase(ALT)  
and Serum aspartate transaminase (AST)

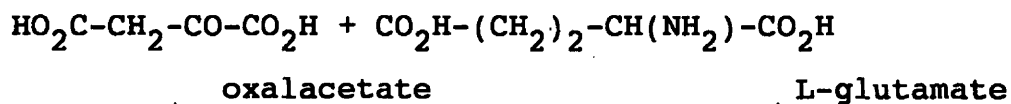
The test is performed in the biochemistry department at NLBTC using reagents supplied by Diagnostica Merck (F.R.Germany), using the Eppendorf Epos Analyzer at 37°C.

(i) The principle of the test is as follows:

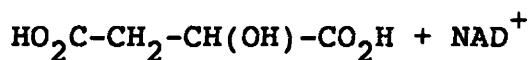
(a) AST



AST ↓↑

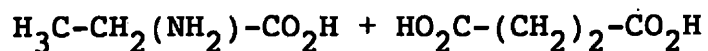


mDH ↓ ↑ NADH



L-malate

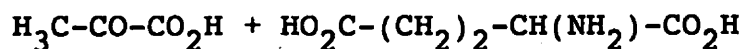
(b) ALT



L-alanine

2-oxoglutarate

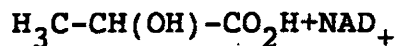
ALT ↓ ↑



Pyruvate

L-glutamate

LDH ↓ ↑ NADH



L-lactate

The rate of decrease in absorbance at 340nm is directly proportional to the concentration of AST or ALT in the serum sample.

Normal ranges:

	AST	ALT
male	4-45 iu/l	4-52 iu/l
female	4-30 iu/l	4-29 iu/l

### Test Procedure.

Merkotest A reagents supplied by BDH are used in conjunction with the Eppendorf Epos Analyzer 5060 to photometrically determine the concentrations of ALT and AST in serum samples .

Reagent 1 for both assays is prepared by emptying the appropriate substrate solution (L-alanine for ALT, L-aspartate for AST) into the corresponding NADH enzyme mixture. After standing for 10 minutes, solution is assisted by gentle mixing.

Reagent 2 (2-oxoglutarate solution) is the same for both assays and requires no preparation.

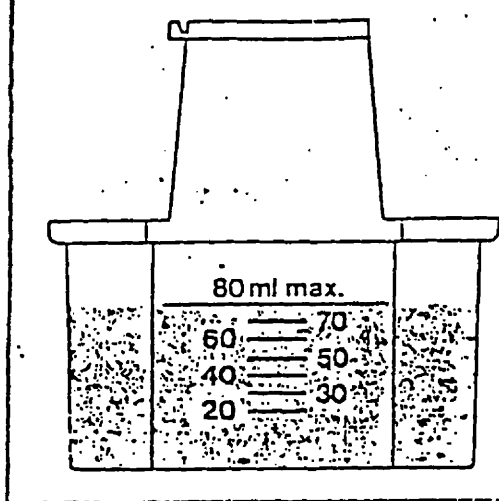
The R1 cup (maximum volume  $80\text{cm}^3$ ) is filled with reagent 1 and placed in position on the Epos. (see diagram).

The R1 cover is prepared by filling C1 with internal control serum and C2 with Wellcome control serum. Cleaner solution (BDH) is placed in the well provided and the cover secured with clear film. It is locked into position on the R1 cup.

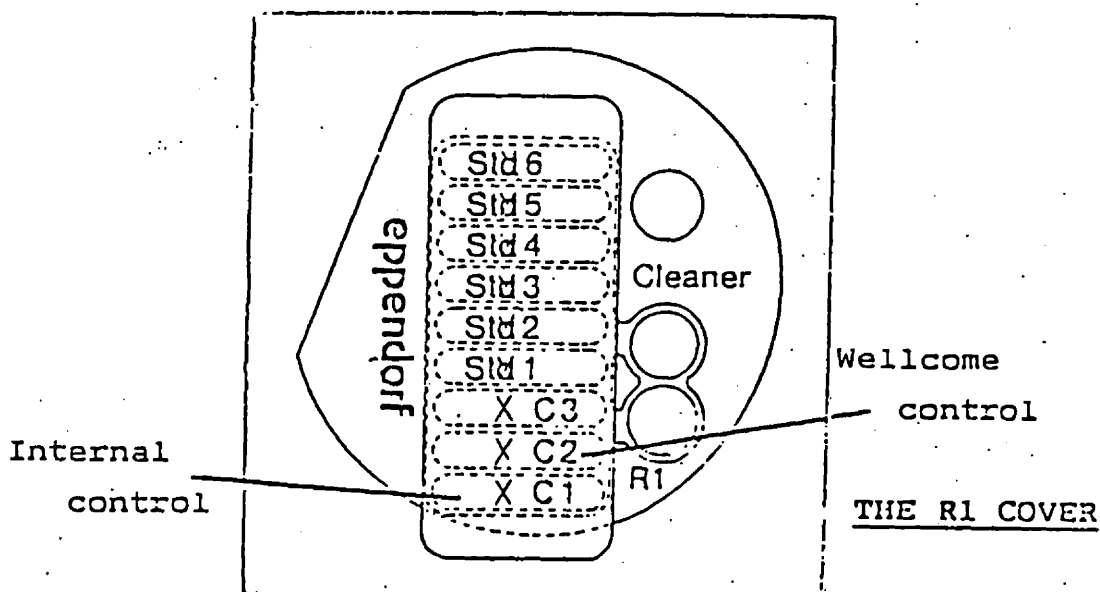
Reagent 2 is poured into the R2 container and placed in position on the Epos.

The appropriate assay is selected and the required information entered. The Epos will perform the assay giving a printout of results. Any samples with enzyme values too high to be measured will be automatically diluted and remeasured by the machine.

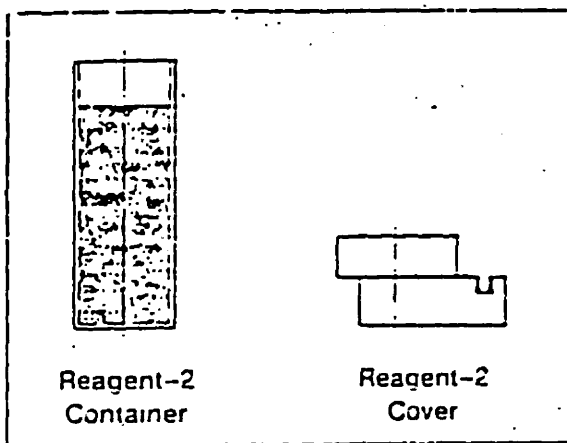
Any results which are outside the normal assay range may be repeated.



THE R1 CUP



THE R1 COVER



Reagent-2  
Container

Reagent-2  
Cover

Figure 45.  
The EPOS analyser.



## Appendix 7: Reverse passive haemagglutination test for alpha-foetoprotein.

The reagents are manufactured by Fujizoki of Japan. Fixed chicken erythrocytes sensitized with mouse monoclonal anti-AFP antibody are agglutinated in the presence of AFP in serum, plasma or ascites.

### Test procedure.

2 drops (0.05 ml) of absorbing diluent are placed in each well of row 1, and 1 drop (0.025 ml) in each of wells 2 to 12 of a microtitration plate, using a calibrated pipette dropper.

5 microlitres of test serum is added to each well of row 1 using a micropipette.

Doubling dilutions are made from row 1 to row 12 using a micropipette.

1 drop (0.025 ml) of control (unsensitised) cells is added to each well of row 1 and 1 drop (0.025 ml) of test (sensitised) cells are added to rows 2 to 12 of the micrototration plate, using the droppers provided.

The plate is then rotated vigorously for 1 minute and incubated horizontally at room temperature for 1 and a half hours and read for haemagglutination.

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